The role of cholecystokinin receptors in the growth of human pancreatic cancer.

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THE ROLE OF CHOLECYSTOKININ RECEPTORS IN THE GROWTH OF HUMAN PANCREATIC CANCER CELLS

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But seek first His kingdom and His righteousness and all these things will be given to you as well.

(Matthew 6:33)

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ABSTRACT

Experimental evidence suggests that growth of the exocrine pancreas is stimulated by many hormones including cholecystokinin and gastrin. The important role of these growth factors in cancer is now being recognised and their analogues or their antagonists are being analysed as possible therapeutic agents for neoplastic diseases. Pancreatic cancer by virtue of its resistance to radiation and chemotherapy, is an appropriate neoplasm in which to explore the applicability of cholecystokinin/gastrin receptor (CCK-R) antagonists in the management of this malignant disease. This thesis entails molecular, cellular and *in vivo* studies in order to determine the role of cholecystokinin/gastrin receptors in the growth of human pancreatic cancer.

The molecular studies show the 7 human pancreatic cancer cell lines examined expressed both CCK-A (CCK-AR) and CCK-B receptors (CCK-BR) but at very low levels. This was in contrast to high levels of expression found in the human gall bladder (CCK-A), human gastric mucosa (CCK-B) and the mouse fibroblast cell line NIH3T3 transfected with the human CCK-BR (NIH3T3CCK-BR). Furthermore, cell culture studies on 2 human pancreatic cancer cell lines (BxPc-3 and Mia PaCa-2), showed a lack of growth response to CCK-R agonists, gastrin (nsG-17) and cholecystokinin (sCCK-8) while NIH3T3CCK-BR showed growth stimulation. Radioligand binding assays showed that the NIH3T3CCK-BR cells had approximately 12.5 fold more CCK-BR than the Mia PaCa-2 cells. Molecular studies using semi-quantitative RT-PCR showed no difference in CCK receptor expression in 4 cancers compared with their matched normal pancreas.

In vivo studies on xenografted Mia PaCa-2 cells in nude mice indicate that treatment with nsG-17 or sCCK-8 have no significant effect on tumour mass, DNA or protein content compared with the control group. Moreover treatment with the CCK-R antagonists tested were unsuccessful in reducing tumour growth.

The discovery of a novel serine/threonine kinase isolated from two human pancreatic cancers which is over-expressed in pancreatic cancers, normal pancreas and in human pancreatic cancer cell lines may prove to be important in the development of this disease and a potential target for the diagnosis and/or treatment of pancreatic cancer.

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CONTENTS

MATERIALS AND METHODS
(Chapter 2-6)

CHAPTER 6 Elucidation of novel protein kinases in pancreatic cancer

RESULTS AND DISCUSSION
(Chapter 7-11)

ABBREVIATIONS

AACN	Acidophilic atypical acinar cell nodules
aFGF	Acidic fibroblast growth factor
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BOP	*N*-nitroso (2-oxopropyl) amine
cAMP	Cyclic 5'-adenosine monophosphate
CCK	Cholecystokinin
CCK-4	Cholecystokinin tetrapeptide
CCK-8	Cholecystokinin octapeptide
CCK-AR	Cholecystokinin receptor subtype A
CCK-BR	Cholecystokinin receptor subtype B
CCK-R	Cholecystokinin receptor
CDK	Cyclin dependent kinase
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic 5'-guanosine monophosphate
CNS	Central nervous system
CT	Computed tomography
C-terminal	Carboxyl terminal
CTP	Cytidine triphosphate
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
ddH_2O	Double distilled water
DAG	Diacyl glycerol
DCC	Deleted in colorectal cancer
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
DPC4	Deleted in pancreatic cancer, locus 4
dNTP	Deoxynucleoside triphosphate
ECL	Enhanced chemiluminescence

EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ERCP	Endoscopic cholangiopancreatography
EtBr	Ethidium bromide
EUS	Endoscopic ultrasonography
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
G-6	Gastrin hexapeptide
G-17	Gastrin heptadecapeptide
GAP	GTPase activating proteins
GAPDH	Glyceraldehyde-phosphate -dehydrogenase
GDP	Guanosine diphosphate
GI tract	Gastrointestinal tract
gly-G17	glycine-extended gastrin heptadecapeptide
GNEF	Guanine nucleotide exchange factor
G protein	Guanine nucleotide-binding protein
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor bound 2
GRP	Gastrin releasing peptide
GTP	Guanosine triphosphate
HGF	Hepatocyte growth factor
IAPP	Islet amyloid polypeptide
IGF	Insulin-like growth factor
IGF-IR	Insulin-like growth factor-I receptor
IORT	Intraoperative radiation therapy
IP_3	Inositol triphosphate
IPTG	Isopropyl-β-thiogalactopyranoside
IRS-1	Insulin receptor substrate-1
JAK	Janus kinase
kb	Kilobase pair

kDa	Kilodalton
MAPK	Mitogen activated protein kinase
MCC	Mutated in colorectal cancer
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MOPS	(3-[N-Morpholino] propanesulphonic acid)
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
nsG-17	Nonsulphated gastrin heptadecapeptide (gastrin I)
NaH_2PO_4	Sodium dihydrogen orthophosphate
NaOH	Sodium hydroxide
NF1	Neurofibromatosis 1
NH_2 terminal	Amino terminal
OD	Optical density
OLB	Oligo labelling buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Pancreaticobiliary diversion
Pi	Inorganic orthophosphate
PI-3-kinase	Phosphatidylinositol-3-kinase
PLC	Phospholipase C
PreproCCK	Preprocholecystokinin
ProCCK	Procholecystokinin
PY20	Phosphotyrosine antibody
Ras	Rous sarcoma virus
RER	Rough endoplasmic reticulum
RNase	Ribonuclease
RNP assay	Ribonuclease protection assay
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse transcription-polymerase chain reaction
sG-17	Sulphated gastrin heptadecapeptide (gastrin II)
sCCK8	Sulphated cholecystokinin octapeptide
SCID	Severe combined immune deficient

SDS	Sodium dodecyl sulphate
SoS	Son of sevenless
SSC	Sodium chloride, sodium citrate
SSTR	Somatostatin receptor
STAT	Signal transducers and activators of transcription
Taq	*Thermus aquaticus*
TBE	Tris borate EDTA
TBS	Tris buffered saline
TE	Tris EDTA
TEMED	N,N,N'N', tetramethylethylenediamine
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
TRIS	Tris (Hydroxymethyl) Aminomethane
TTP	Thymidine triphosphate
Tween 20	Polyoxyethylene (20) sorbitan monolaurate
Tyr	Tyrosine residue
UTP	Uridine triphosphate
UV	Ultraviolet
V	Volts
v/v	Volume/volume
w/v	Weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

CHAPTER 1

1.1 CHOLECYSTOKININ

Cholecystokinin (CCK) is among the most well characterised of the peptides that were initially discovered in the gut (Ivy & Oldberg, 1928) and subsequently localised in the central nervous system (Beinfeld *et al.*, 1981, Beinfeld & Palkovits, 1982). CCK extracted from porcine intestine, was originally identified as a 33 amino acid peptide (Jorpes & Mutt, 1966; Mutt & Jorpes, 1968).

1.1.1 Molecular characterisation

The chemical structure of CCK is complicated by the natural occurrence of multiple molecular forms that range from 4 to 58 amino acids (CCK-4 to CCK-58) of which sulphated CCK-8 (sCCK-8) is probably the dominant form (Anderson & Dockray, 1988). Species-specific molecular variants of the CCK amino acid sequences have been identified which are identical at the pentapeptide carboxyl terminus in all species studied. This terminus is also present in the gastrointestinal hormone gastrin (Mutt, 1980). The pentapeptide appears to be the minimum sequence required for biological activity (Crawley *et al.*, 1984; Mutt, 1980, see Figure 1.1). The 33 amino acid sequence of CCK-33 and its 8 amino acid C-terminal have been demonstrated in pig, rat, chicken, dog and man (Eysselein *et al.*, 1984; Fan *et al.*, 1987; Maton *et al.*, 1982; Rehfeld,1978a). A prominent feature of the structure of CCK-33 is the presence of a sulphated tyrosine at position 27. The sulphated C-terminal octapeptide (sCCK-8) possesses a high degree of biological activity (Ondetti *et al.*, 1970; Williams, 1982), and is approximately 1500 times more potent than its non-sulphated counterpart (nsCCK-8, Patel & Spraggs, 1992) in contracting the longitudinal muscle of the guinea-pig ileum. The activity of sCCK-8 in stimulating pancreatic growth is thought to be equipotent to that of sCCK-33 (Williams, 1982; Anderson & Dockray, 1988). The tetrapeptide (CCK-4) is approximately 22,000 times less potent than sCCK-8 overall (Williams, 1982; Patel & Spraggs, 1992). Neither the C-terminal tripeptide nor the deaminated tetrapeptide appear to have any biological activity.

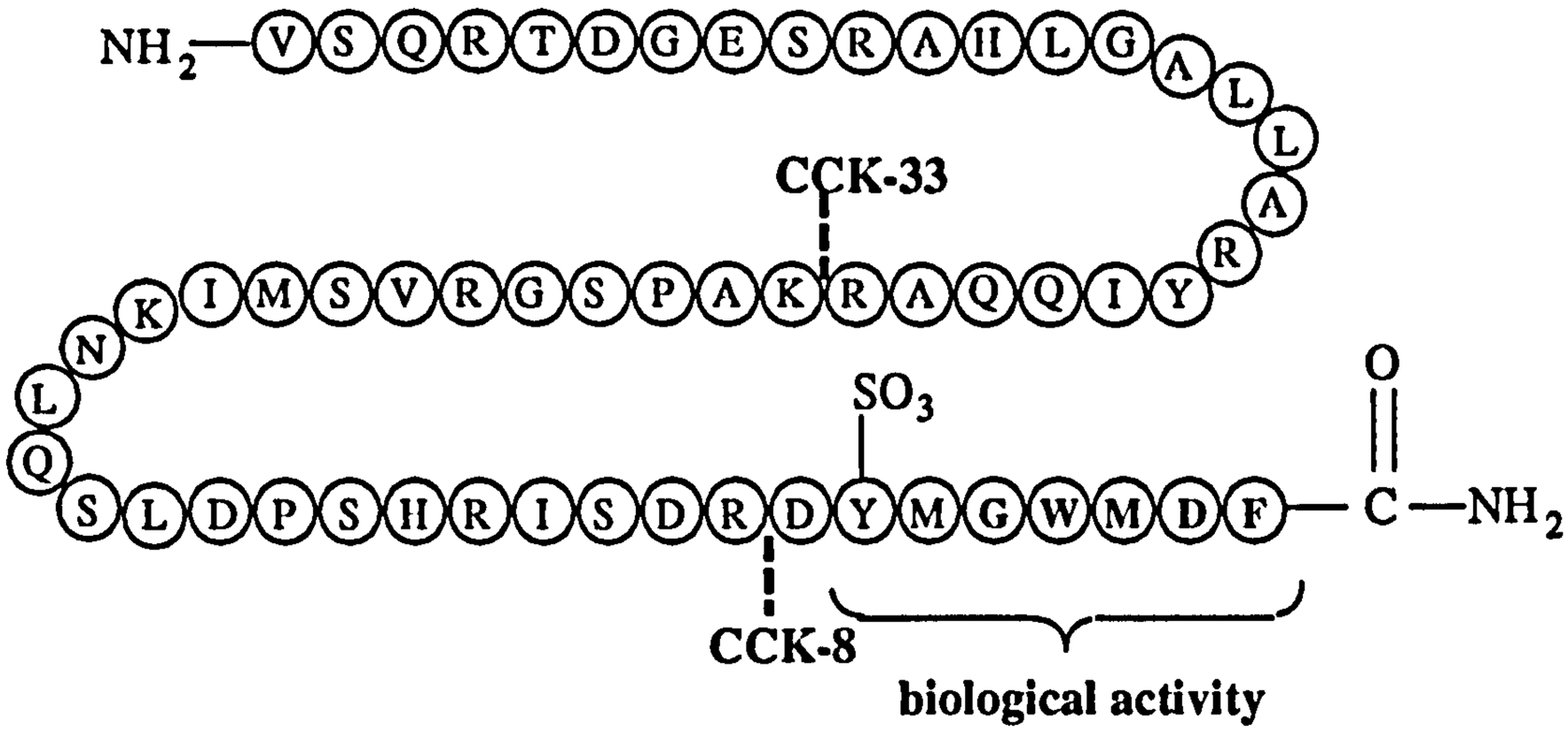


Figure 1.1 Structure of CCK-58. The C-terminal pentapeptide sequence is important for the biological activity of the peptide (indicated are the sequences for CCK-8 and CCK-33).

1.1.2 Post translational processing of procholecystokinin

1.1.2.1 *Intestinal procholecystokinin (proCCK)*

Although the majority of CCK is synthesised in the central nervous system, an essential portion is synthesised in the endocrine cells of the intestinal mucosa (Rehfeld, 1978a). Moreover, the majority of the CCK in plasma originates from the endocrine I cells of the small intestine, named according to *Wiesbaden classification* of gut endocrine cells (Solcia *et al.*, 1973). I cells have not been shown to contain other gut hormones. ProCCK-derived peptides of different chain lengths (varying from 4-95 amino acids) have been identified from extracts of both the small intestine and brain (Mutt & Jorpes, 1968, 1971; Mutt, 1976).

Cholecystokinin is translated from its mRNA to preprocholecystokinin (preproCCK) in the rough endoplasmic reticulum (RER). In human and rat preproCCK is a 115 amino acid polypeptide that undergoes specific enzymatic cleavage to form the biologically

active hormone. Following translation, the N-terminal pre- (or signal peptide) is removed from preproCCK and the remaining intact proCCK (95 amino acids) is transported to the Golgi apparatus. In the Golgi apparatus proCCK is completely tyrosyl-O-sulphated at three positions (Tyr-77, 92 and 95) and cleaved at multiple processing sites. The endoproteolytic activity continues in small vesicles which carry the processing intermediates to the basal part of the I cells, where the processing continues in the secretory granules (see Figure 1.2). The final step in the synthesis of bioactive CCK peptides occurs during storage and maturation in the secretory granules that contain the precursor for the two enzymes necessary for amidation, which removes glyoxylate from the immediate precursors (the glycine extended CCKs), to complete the synthesis of bioactive α-carboxyamidated peptides.

As a result of the biosynthetic pathway, the intestinal I cells release a heterogeneous mixture of proCCK products from the mature secretory granules. A small percentage are non-amidated precursors. The amidated CCKs constitute a mixture of the longest possible bioactive product of proCCK, *i.e.*, CCK-83 (Eberlein *et al.*, 1992), as well as the intermediate sized CCK-58, -39, -33, and -22 (Eng *et al.*, 1984; Mutt and Jorpes, 1968; Mutt, 1976; Rehfeld, 1978b) and the short CCK-8 and CCK-5 (Dockray *et al.*, 1978; Rehfeld & Hansen, 1986; Shively *et al.*, 1987). The distribution and release patterns vary widely among species (Cantor & Rehfeld, 1989; Eberlein *et al.*, 1988).

1.1.2.2 *Extra-intestinal procholecystokinin*

Neurones in all regions of the brain synthesise CCK peptides (as in the I cells), though cerebellar neurones do so only in the foetal stage (Larsson and Rehfeld, 1979a; Rehfeld, 1992). In addition, cholecystokinin coexists within the same neurone as other neurotransmitters at many sites such as dopamine and substance P (Hokfelt *et al.*, 1980; Skirboll *et al.*, 1982). CCK peptides are widely expressed in peripheral neurones, primarily in the gastrointestinal tract but also in the genitourinary tract (Larsson and Rehfeld, 1979a). Low levels of expression have been found in pituitary corticotrophs (Rehfeld, 1986), in thyroid C cells (Rehfeld *et al.*,1990), in the adrenal medulla (Bardram *et al.*, 1989), in the bronchial mucosa (Ghatei *et al.*, 1982) and in spermatogenic cells of certain species (Persson *et al.*, 1989).

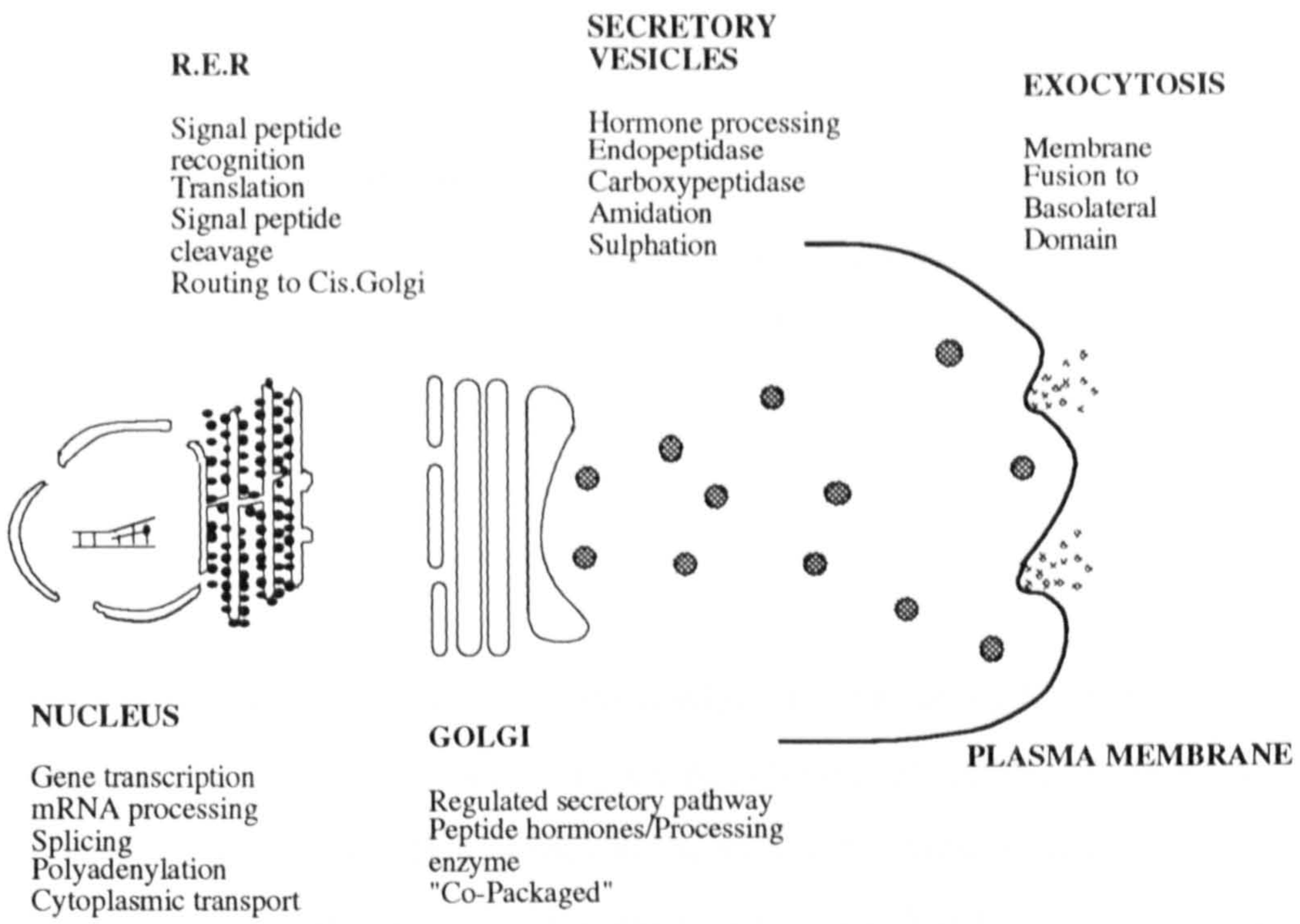


Figure 1.2 A model showing the organelles and some of the biochemical reactions responsible for the synthesis, processing and targeting for secretion of GI peptide hormones.

1.1.3 Biological Actions

1.1.3.1 *Gall bladder contraction*

CCK was originally discovered because of its ability to stimulate gallbladder contraction. Convincing evidence that endogenous CCK is critical for gallbladder contraction has recently been provided by the use of specific CCK receptor antagonists. Sufficient doses of antagonist have consistently demonstrated complete blockade of either food or CCK-stimulated gallbladder contraction (Liddle *et al*., 1989; Niederau *et al*., 1989). Physiological levels of CCK in blood that occur after a meal are sufficient to account for postprandial gallbladder contraction (Liddle *et al*., 1985). In addition to effects on the gallbladder, CCK stimulates hepatic secretion of bicarbonate into bile (Shaw & Jones, 1978) and induces relaxation of the sphincter of Oddi (Lin, 1975).

Thus, after a meal CCK co-ordinates many aspects of bile delivery to the duodenum.

1.1.3.2 *Lower oesophageal sphincter*

Several gastrointestinal hormones including CCK have been shown to relax the lower oesophageal sphincter (Resin *et al.*, 1973). CCK appears to decrease lower oesophageal sphincter pressure through a nonadrenergic noncholinergic, neuronal mechanism (Dodds *et al.*, 1981).

1.1.3.3 *Gastric emptying*

In most species studied, CCK slows the rate at which food empties from the stomach. (Debas *et al.*, 1975). This delay occurs by CCK-induced relaxation of the proximal part of the stomach and constriction of the pyloric sphincter (Anuras *et al.*, 1974; Chey *et al.*, 1970). The ability of CCK to delay gastric emptying has been shown to occur with even low levels of circulating hormone (Liddle *et al.*, 1986a; Debas *et al.*, 1975). The identification of CCK receptors on the pyloric sphincter provide strong evidence that CCK directly effects the gastric musculature (Smith, *et al.*, 1984).

1.1.3.4 *Intestinal motility*

In the intestine, CCK has been shown to stimulate motor activity and decrease intestinal transit time (Bertaccini & Agosti 1971; Levant *et al.*, 1974). CCK appears to affect bowel motility by two routes. First, it has been shown to release acetylcholine from parasympathetic postganglionic neurones (Vizi *et al.*, 1973) and second, to act directly on smooth muscle of the small bowel to stimulate contraction (Yamagishi & Debas, 1978).

1.1.3.5 *Gastric acid secretion*

CCK peptides are weak stimulants of basal acid secretion in most species but inhibit stimulated acid secretion. In the cat, CCK stimulates acid secretion to the same maximum achieved by gastrin and has no inhibitory action (Way, 1971). Observations that injection of high amounts of CCK reduced gastric acid led to the hypothesis that CCK may function as an enterogastrone in humans and dogs. Enterogastrones are hormonal substances released by fat in the upper small intestine that cause inhibition of

gastric acid secretion. The CCK-AR antagonist, devazepide, was found to potently reverse CCK-induced inhibition of meal-stimulated gastric acid secretion in dogs and significantly reverse acid inhibition produced by intestinal perfusion with fat (Lloyd *et al.*, 1992).

1.1.3.6 *Pancreatic effects*

CCK has been shown to stimulate growth of the pancreas when administered to animals (Brants & Morisset, 1976). This effect appears to be a physiological action of CCK, since feeding trypsin inhibitors stimulated pancreatic growth while treatment of animals with CCK receptor antagonists produced pancreatic atrophy (Niederau *et al.*, 1987).

The administration of glucose or amino acids into the intestine results in greater insulin secretion than the same amount of nutrient given intravenously (Hampton *et al.*, 1986). CCK receptors have been identified on β cells of rat pancreatic islets (Sakamoto *et al.*, 1985) indicating that islet β cells have CCK receptors that are linked to the stimulation of insulin release. In humans, although exogenous CCK has been shown to potentiate amino acid-stimulated insulin release (Rushakoff *et al.*, 1987), CCK alone does not significantly modify insulin secretion since CCK receptor (CCK-R) blockade with specific CCK-A receptor antagonists did not affect insulin levels. In addition to insulin; CCK also potentiated the release of other islet cell hormones, including glucagon and pancreatic polypeptide (Schmid *et al.*, 1989). The precise role of CCK in the regulation of insulin and glucagon secretion remains unclear.

Preparations of isolated pancreatic acini *in vitro* have provided an excellent model for studying pancreatic secretion on a cellular level. Extremely low concentrations of CCK are needed to stimulate secretion of most pancreatic enzymes (Kerstens *et al.*, 1985). Although amylase is the most abundant pancreatic enzyme secreted, many other pancreatic enzymes are also secreted in parallel but in comparison CCK has little effect on their release (Nakajima *et al.*, 1973). This differential effect of CCK on enzyme secretion is referred to as nonparallel secretion. In addition to stimulating pancreatic enzyme secretion, CCK has direct effects on enzyme biosynthesis. In particular, CCK

has been shown to increase the pancreatic content of serine proteases relative to amylase (Leroy *et al.*, 1971). CCK is also a weak stimulant of bicarbonate secretion (You *et al.*, 1983).

1.1.3.7 *Satiety*

The discovery by Gibbs *et al.* (1973) that CCK decreases food intake in rats led to intensive investigations into the mechanisms by which CCK induces satiety. In rats, CCK induces the typical behavioural pattern of satiety such as grooming and exploration as well as a reduction in food intake (Antin *et al.*, 1975). The ability of peripherally administered CCK to produce satiety, appeared to be mediated by the vagus nerve since CCK had no effect on satiety when given to vagotomised animals (Smith *et al.*, 1981). Investigations on rhesus monkeys indicated that the satiety effect of peripherally administered CCK was due to its ability to inhibit gastric emptying (McHugh & Moran, 1986). However, studies in baboons suggested that effects on gastric emptying could not account for all the satiety effects of CCK (Stein *et al.*, 1986). Thus, the exact mechanism(s) by which vagal afferents signal satiety within the brain are unknown. Research determining whether the levels of CCK in the blood are sufficiently high to produce satiety have been inconclusive. In dogs, the amount of CCK required to cause satiety was greater than that necessary to stimulate gall bladder contraction (Pappas *et al.*, 1985). Later studies in pigs immunised against CCK had increased food intake and accelerated weight gain compared to non-immunised animals (Pekas & Trout, 1990; Pekas, 1991). Studies with CCK-AR specific antagonists showed that CCK-AR blockade increased food intake in pigs (Ebenezer *et al.*, 1990) and decreased satiety in humans (Wolkowitz *et al.*, 1990) and CCK-BR antagonists had no effect. These studies with CCK-R antagonists provide evidence that endogenous CCK plays a role in regulating satiety.

1.1.4 Release

CCK is released into the blood from the duodenum during ingestion of a meal. The CCK levels gradually increase to a maximum at 10-45 minutes following the start of a meal and then gradually decline, but remain elevated upto 3 to 5 hours after eating.

Food components including protein digestates and fat are stimulants of CCK secretion (Meyer & Jones, 1974; Liddle *et al.*, 1985). The amino acids tryptophan and phenylalanine appear to be the most potent stimulants of intestinal CCK (Go *et al.*, 1970; Konturek *et al.*, 1973) and carbohydrate in the form of glucose is also a weak stimulant of CCK (Liddle *et al.*, 1985). However, there are clear species differences in the CCK responses to different food components (Liddle *et al.*, 1986b). The exact mechanisms by which food stimulates CCK secretion have yet to be elucidated.

Recently, two naturally occurring trypsin-sensitive peptides have been shown to act within the lumen of the intestine to stimulate CCK release (Iwai *et al.*, 1987; Miyasaka *et al.*, 1989). Monitor peptide, a 61 amino acid protein is produced by the pancreas and secreted into pancreatic juice. Upon reaching the intestine, monitor peptide stimulates CCK release. In contrast, another "CCK releasing factor" has been partially characterised from the intestine (Lu *et al.*, 1989; Miyasaka *et al.*, 1989). This factor is secreted by the intestine into the gut lumen under basal conditions.

At present it is not known which of these factors is the most important for controlling CCK secretion but it is known that multiple mechanisms exist to ensure proper regulation of CCK release such as bombesin and vagal stimulation (Walsh, 1987). Stimulants responsible for the release of CCK from the brain and the male reproductive system are yet unknown.

1.1.5 Metabolism

Peptides shorter than CCK-8 are broken down much faster than the larger forms. Plasma CCK is mainly degraded by aminopeptidase (Deschodt-Lanckman *et al.*, 1981, 1983). CCK peptides with sulphated tyrosine are degraded more slowly than their non-sulphated counterparts. Several types of enzymatic activity in tissue are able to degrade CCK-8 including membrane bound aminopeptidases (Deschodt-Lanckman, 1982). It is well known from studies with gastrin fragments that the pentapeptide and tetrapeptide common to gastrin and CCK are almost removed from the portal blood during a single pass through the liver (Doyle *et al.*, 1984).

1.2 GASTRIN

Gastrin is a gastrointestinal peptide hormone which was first extracted by Edkins in 1905 and the protein sequence was independently determined later in 1964 (Anderson *et al.*, 1964; Gregory & Tracy, 1964).

1.2.1 Molecular characterisation

There are a number of gastrin peptides which range from 5 to 34 amino acids in sequence (Walsh & Grossman, 1975) arising from a larger parent polypeptide (Walsh, 1987). The most abundant form in the duodenum is the 34-amino acid (G-34), whereas the heptadecapeptide G-17 is more abundant in the antrum. About half of G-17 and G-34 molecules in the antrum and duodenum have a sulphated tyrosine residue which is located in the sixth position proximal to Phe-NH_2 (Walsh, 1987). Sulphated forms of gastrin are also known as gastrin II. The most abundant form of gastrin in the blood corresponds to G-34. About two-thirds of circulating gastrin in the basal state (before a meal) and about half in the postprandial state is G-34.

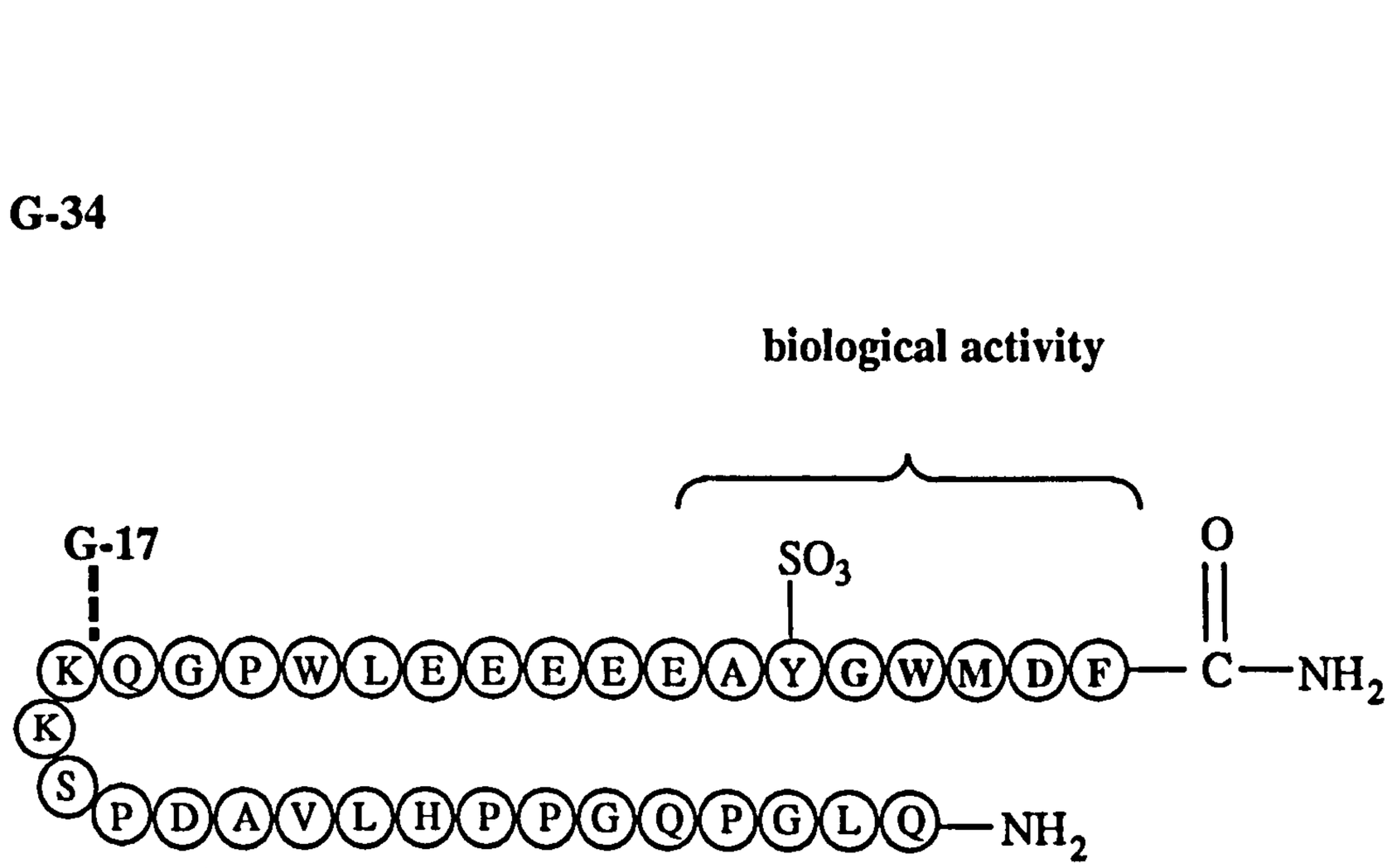


Figure 1.3 Structure of G-34 . The C-terminal pentapeptide sequence is important for the biological activity of the peptide (the sequence of G-17 is indicated).

The remainder is G-17 and small amounts of G-14, which is a smaller fragment of gastrin also known as minigastrin (Gregory *et al.*, 1979). The hexapeptide G-6 is the smallest gastrin fragment isolated from the stomach (Gregory *et al.*, 1983). Larger forms of gastrin have not yet been characterised structurally. Species differences have been found in gastrins from different mammals (Reeve *et al.*, 1981; Bonato *et al.*, 1986; Jiang *et al.*, 1988).

1.2.2 Post-translational processing of progastrin

1.2.2.1 *Antral progastrin*

The majority of gastrin is synthesised and secreted by endocrine cells in the antral mucosa of the stomach (Larsson, 1980; Rehfeld, 1981). These cells are known as G cells. These G cells can be distinguished from CCK-containing cells by specific patterns of antibody reactivity and by ultrastructural appearance. Some gastrin cells are found in the adult duodenal mucosa (Buchan *et al.*, 1979). However, gastrin biosynthesis studies have so far focused on antral tissue (Brand *et al.*, 1984; Hilsted & Rehfeld, 1987).

Gastrin is translated from its mRNA to preprogastrin in the rough endoplasmic reticulum (RER). Following translation the N-terminal signal peptide is removed from preprogastrin and the remaining intact progastrin (95 amino acids) is transported to the Golgi apparatus. In the Golgi apparatus progastrin is tyrosyl-O-sulphated (66) and cleaved at multiple processing sites. From the Golgi apparatus, network vesicles carry the intermediates of progastrin towards the basal part of the G cells, where the peptides are stored in characteristic secretory granules (Hakanson *et al.*, 1982; Larsson & Rehfeld, 1979b). It is thought that the endoproteolytic trypsin-like and exoproteolytic carboxypeptidase E-like processings (as well as the subsequent glutamyl cyclisation, corresponding to the N-termini of G-34 and G-17) continue during the transport from the Golgi to the early secretory granules. The final processing step in the synthesis of gastrin occurs during storage and maturation in the secretory granules. The amidation enzymes in the granules remove glyoxylate from the immediate precursors (the glycine-extended gastrins), to complete the synthesis of bioactive α-carboxyamidated peptides.

Amidation is a crucial all-or-none activation process which is carefully controlled (Hilsted *et al.*, 1986, 1988).

The results of this biosynthetic pathway release a heterogeneous mixture of progastrin products from the secretory granules (G71, 34, 17, 15, 14, 7 and 4). A small percentage are non-amidated precursors, mainly glycine-extended gastrins. In humans >95% are α-amidated bioactive gastrins, of which the longest molecular form is G-71. Thus of the amidated gastrins 90% are G-17, 5% are G-34 and the rest a mixture of G-71, G-15, G-14 and short C-terminal hepta- or tetrapeptide amide fragments (Gregory *et al.*, 1983; Hilsted & Hansen, 1988; Rehfeld & Larsson, 1979; Yalow & Berson, 1970). Approximately half of the amidated gastrins are tyrosyl-sulphated (Hilsted & Rehfeld, 1987).

Due to gross differences in metabolic clearance rates, the distribution of gastrins in peripheral plasma changes so that larger gastrins with their long half-lives predominate over G-17 and shorter gastrins (Jensen, *et al.*, 1989).

1.2.2.2 *Extra-antral progastrin*

The gastrin gene is expressed at the peptide level in several cell types other than the G cells. Quantitatively, the gastrin contribution in plasma from other cells is very small. This is mainly because the secretion seems to serve local purposes rather than a general endocrine function and also because the biosynthetic processing is cell specific. Expression of progastrin and its products has been detected in the distal small intestine (Buchan *et al.*, 1979), colon, (Luttichau *et al.*, 1993), in endocrine cells in the foetal and neonatal pancreas (Bardram *et al.*, 1990), in spermatogenic cells (Schalling *et al.*, 1990), in a few cerebellar and vagal neurones (Rehfeld, 1991; Uvnas-Wallensten *et al.*, 1977) and in the bronchial mucosa (Rehfeld *et al.*, 1989). The concentrations and synthesis of gastrin in the extra-antral tissues are far below those of the antrum and the precise function of gastrin synthesised outside the antroduodenal mucosa is largely unknown. However, one important function is in the paracrine or autocrine regulation of malignant growth (Hoosein *et al.*, 1988).

Although it is possible that extra-antral synthesis of gastrin is without function in the adult organism, recognition of the phenomenon has considerable clinical relevance. Hence, tumours originating from tissues and cells that normally express the gastrin gene even at low level may produce gastrin in lethal amounts.

1.2.3 Biological Actions

1.2.3.1 *Acid secretory effects*

Gastrin has a wide range of actions on epithelial and smooth muscle targets in the gastrointestinal tract. Gastrin plays an important role in the regulation of meal-stimulated gastric acid secretion (McGuigan *et al.*, 1971; Walsh & Grossman, 1975). Large fragments of gastrin that contain the C-terminal tetrapeptide amide sequence, including G-34, G-17, and G-14 have similar acid-stimulating potency (Carter *et al.*, 1979; Eysselein *et al.*, 1984). Both sulphated and non-sulphated forms of G-17 and of G-14 have similar acid stimulating potency. The primary target of circulating gastrin may be the histamine-containing gastric enterochromaffin-like cell, the parietal cell or both (Soll *et al.*, 1984; Chuang *et al.*, 1992; Sandvik & Waldum, 1991; Ruiz &Michelangeli, 1986).

1.2.3.2 *Trophic effects*

A potentially important role of gastrin is the regulation of mucosal growth, especially of the acid-secreting mucosa of the stomach (Johnson, 1976). Most cell division occurs in the area of the mucous neck cells. From here cells migrate to the surface of the gland where they become parietal cells. The origin of the chief and enterochromaffin-like cells is currently unknown. When gastrin is released it passes into the circulation to the oxyntic mucosa where it stimulates DNA synthesis (Enochs & Johnson, 1977) and proliferation of the oxyntic mucosal cells (Crean *et al.*, 1969), particularly the parietal cells and the ECL cells. Resection of the antrum (the site of gastrin secretion) results in atrophy of the gastric mucosa (Johnson, 1988). This atrophy can be reversed by the administration of gastrin. CCK in high doses appears to be a competitive inhibitor of the trophic effects of gastrin in the stomach (Johnson, 1977). Trophic effects of gastrin have also been found in the colon mucosa (Dembinski & Johnson, 1979).

1.2.4 Release

Food components, namely glucose and fats, are weak stimulants of gastrin release (Walsh, 1987; Walsh, 1992). Gastrin is mainly released in response to peptides, amino acids (phenylalanine and tryptophan) and calcium in the gastric lumen, by the activation of nervous reflexes, and by catecholamines and bombesin circulating in blood (Walsh & Grossman, 1975). Food components, especially small peptides and amino acid fragments resulting from the digestion of protein also cause gastrin release (Taylor *et al.*, 1982). Gastrin is also released by vagal stimulation mediated by cholinergic mechanisms that may be induced by the sight, smell and taste of food (Nilsson *et al.*, 1972). Gastrin release is inhibited by a low pH in the stomach, by several peptides including somatostatin and by certain prostaglandins (Walsh, 1987; Walsh, 1988).

1.2.5 Metabolism

The rates at which various forms of gastrin disappear from the circulation and the sites of removal have been studied more thoroughly than the mechanisms for inactivation. The liver does not appear to play an important role in clearance of larger gastrins present in the circulation, but it removes short gastrin fragments such as the tetrapeptide and pentagastrin (Strunz, *et al.*, 1978). The kidney may play a major role in clearance of G-34 (Loly *et al.*, 1982). The kidney tubule contains enzyme activity that cleaves the C-terminal dipeptide from G-4 (Walsh & Laster, 1973), however it is still unclear if this enzyme acts on the larger gastrin molecules.

1.2.6 Mode of cellular release of CCK and Gastrin

Different types of cells release CCK and gastrin in different manners. CCK and gastrin are now known to be released by five different routes. i) Classical endocrine secretion, where secretory granules of the endocrine I and G cells in the gastrointestinal mucosa empty their peptides into surrounding capillaries after appropriate stimulation. ii) Peptides in neurones are released from synaptosomal vesicles in the nerve terminals to the receptors of adjacent target cells *i.e.* neurotransmitter or neurocrine release. iii) It has been shown that there are gastrin-producing paracrine cells in the small intestinal mucosa (Larsson, 1980). These cells carry gastrin granules through cytoplasmic extensions to specific target cells in the neighbourhood. iv) Autocrine secretion

whereby the trophic peptides bind to their specific receptors in the membranes of cells in which they are synthesised. The growth of colon cancer *in vitro* (Hoosein *et al.*, 1988, 1990) and pancreatic cancer cells (Blackmore & Hirst, 1992; Seva *et al.*, 1990a) has been shown to be stimulated by autocrine secretion. v) In spermatozoa, the CCK or gastrin peptides are carboxyamidated and are concentrated in the acrosome. The peptides are released from the spermatozoon by contact with the jelly coat of the egg and are subsequently bound to the receptors in the egg membrane.

1.3 CHOLECYSTOKININ AND GASTRIN RECEPTORS

The cholecystokinin and gastrin families of peptides act as hormones and neuropeptides on central and peripheral CCK receptors to mediate their physiological responses. CCK and its receptors are widely distributed in the central nervous system (CNS) and contribute to the regulation of satiety, anxiety, analgesia and dopamine-mediated behaviour. Although the wide distribution, myriad functions and reported pharmacological heterogeneity of CCK receptors (CCK-R) might suggest a large number of subtypes, application of modern molecular biological techniques has so far identified only two CCK receptors. These are the CCK-A and CCK-B receptors that mediate the actions of CCK and gastrin. Gastrin receptors have been found to be identical to the CCK-B receptor (Kopin *et al.*, 1994).

Receptors for CCK have been pharmacologically classified on the basis of their affinity for the peptide agonists CCK and gastrin and by recently developed subtype specific antagonists (Presti & Gardner, 1993).

1.3.1 Discovery

The CCK-A receptor (CCK-AR) was first characterised on pancreatic acinar cells (Sankaran *et al.*, 1980) and in the same year a second receptor with a different pharmacology was discovered in the brain (Innis & Snyder, 1980) and termed the CCK-B receptor (CCK-BR).

The characterisation of CCK binding sites was conducted using agonists and antagonists. The differences in binding affinities of the various forms of CCK resulted in the elucidation of two subtypes of CCK-R. The CCK-AR is highly selective for sulphated analogues (500- to 1000-fold higher affinity) and the antagonist L-364, 718 (Chang & Lotti, 1986). CCK-BR have similarly high affinity for both the sulphated and non-sulphated peptide analogues of CCK and gastrin (only a 3- to 10-fold higher affinity for sulphated peptide analogues) and the antagonist L-365, 260 (Lotti & Chang, 1989). The receptors were termed the peripheral or alimentary CCK-A receptor subtype and the central or brain CCK-B/gastrin receptor subtypes, respectively.

1.3.2 Distribution and function

1.3.2.1 *Cholecystokinin-A receptors (CCK-AR)*

The CCK-AR has been described on pancreatic acinar cells (Sankaran *et al.*, 1980), gall bladder smooth muscle (Bitar & Makhlouf, 1982), chief and D cells of gastric mucosa (Soll *et al.*, 1984), selected areas of the central and peripheral nervous systems (Moran *et al.*, 1986) and tumour cell lines such as the rat pancreatic tumour cell line, AR42J, (Logsdon, 1986) and the human neuroblastoma cell line CHP 212 (Klueppelberg *et al.*, 1990) where they mediate growth.

CCK-AR stimulation with physiological concentrations of CCK causes pancreatic exocrine enzyme secretion (Sankaran *et al.*, 1980), growth (Zucker *et al.*, 1989), endocrine islet cell secretion of insulin (Rosseti *et al.*, 1987) and pancreatic polypeptide (Liddle *et al.*, 1990). CCK-AR in the stomach mediates secretion of pepsin from gastric chief cells and release of somatostatin from D cells of the gastric mucosa, resulting in inhibition of acid secretion (Lloyd *et al.*, 1992). CCK-AR on smooth muscle cells cause gall bladder emptying (Bitar & Makhlouf, 1982), increased pyloric sphincter tone with delayed gastric emptying and within the intestine decreased small bowel and increased colonic transit time (Meyer *et al.*, 1989). CCK-AR is also present in the sphincter of Oddi (Behar & Bianchini, 1980) and on inhibitory postganglionic neurones in the lower oesophageal sphincter causing relaxation (Rattan & Goyal, 1986).

Although the CCK-AR has been traditionally classified as a peripheral receptor, it is present in selective areas of the central nervous system and peripheral nervous system (vagus nerve), where it mediates the satiety effect of CCK. CCK-AR in the nucleus accumbens regulates dopamine release that has potential implications in human neuropsychiatric diseases such as schizophrenia (Crawley, 1991). The CCK-AR present in the dorsal horn of the spinal cord in primates antagonise opioid analgesia tolerance (Baber *et al.*, 1989) and in the anterior pituitary corticotrophs CCK-AR mediates the release of adrenocorticotrophic hormone and β-endorphin (Kamilaris *et al.*, 1992).

1.3.2.2 *Cholecystokinin-B receptors (CCK-BR)*

The CCK-BR is found on cells throughout the central nervous system (Innis & Snyder 1980), immune cells such as monocytes and T lymphocytes (Lignon *et al.*, 1991) and tumour cell lines such as the AR42J cell line (Lambert *et al.*, 1991) and human leiomyosarcoma cells (Pearson *et al.*, 1989).

CCK acting on the CCK-BR in the anterior nucleus accumbens, unlike the CCK-AR in the posterior nucleus accumbens, has an inhibitory effect on dopamine release. The CCK-BR is also involved in the regulation of anxiety by peripheral and central CCK (Harro *et al.*, 1993). The function of CCK-B receptors on monocytes and T lymphocytes is yet unknown. The CCK-BR present on tumours and tumour derived cell lines stimulates growth. The CCK-BR mediates acid secretion from parietal cells in the stomach (Soll *et al.*, 1984), release of histamine from enterochromaffin-like cells (Nakata *et al.*, 1992) and contraction of smooth muscle cells (Grider & Makhlouf, 1990). CCK-BR is also present on pancreatic acinar cells in dog and guinea pig where it mediates growth but not enzyme secretion (Fourmy *et al.*, 1984; Yu *et al.*, 1990).

The so called "gastrin receptor" which was first characterised on canine parietal cells (Soll *et al.*, 1984) has equally high affinity for sulphated CCK and gastrin and an intermediate affinity for non-sulphated CCK and CCK-4 compared with the CCK-B receptor (Chang *et al.*, 1986; Soll *et al.*, 1984). The basis for suspecting that the gastrin receptor in the stomach and CCK-BR in the brain were distinct types of CCK receptors was due to their different relative affinities for CCK and gastrin-like peptides (Menozzi *et al.*, 1989). However, researchers have subsequently shown that this receptor is the CCK-B receptor: when the canine parietal cell gastrin receptor was expressed in COS cells it had the same affinity for CCK and gastrin displayed by the native CCK-BR in human and guinea-pig (Kopin *et al.*, 1994). Furthermore, high stringency Northern blot analysis utilising a fragment of the gastrin receptor as a probe, showed receptor messenger RNA in the canine gastric parietal cells, pancreas and cerebral cortex. The idea that the gastrin receptor was different from the CCK-BR was also supported by the affinities for the benzodiazepine derived CCK-BR antagonist, L-365, 260 and CCK-AR antagonist, L-364,718. The canine parietal cells showed a

greater affinity for the L-364,718 compound, whereas the CCK-BR in the brain of other species showed a higher affinity for L-365,260 (Lotti & Chang, 1989). The reversal in canine gastrin-receptor affinity for the antagonists has been ascribed to a species specific change of a single nucleotide, resulting in a single amino acid substitution (valine in the rat and human and leucine in the dog) in the sixth transmembrane domain (Beinborn *et al.*, 1993).

1.3.3 CCK receptor structure

CCK receptor structure has been studied primarily by radioligand affinity cross-linking, followed by receptor purification and most recently by the cloning of the receptor cDNA and deduction of the primary amino acid sequence.

1.3.3.1 *CCK-A receptors*

The size and number of covalently cross-linked protein bands vary with the ligand, cross-linking reagent, species and the tissue expressing the CCK receptor (Miller, 1984; Pearson & Miller, 1987; Pearson *et al.*, 1987; Rosenzweig *et al.*, 1983). In rat pancreatic acinar cells, the CCK-AR was found to be a 85-95 kDa NH_2-linked glycoprotein having a 42-44 kDa protein core (Pearson *et al.*, 1987). The rat pancreatic CCK-AR was purified by using ion-exchange and multiple affinity chromatographic separations, followed by chemical/enzyme cleavage. Progressively purified receptor was detected using [^{3}H]L-364,718 binding assay (Wank *et al.*, 1992a). This allowed partial peptide sequencing and the synthesis of degenerate oligonucleotides which were used successfully in cloning the CCK-AR cDNA from a rat pancreatic cDNA library (Wank *et al.*, 1992a). The rat CCK-AR cDNA was further used to clone the human CCK-AR cDNA (de Weerth *et al.*, 1993a). Briefly, a human placenta genomic library was screened using [^{32}P] random primed probe derived from the rat CCK-AR cDNA. Primers were selected from the clone isolated from the human placenta genomic library (non-coding regions of the clone). The selected primers successfully amplified CCK-AR cDNA from the human gall bladder. The sequencing results showed a 428 amino acid protein with a molecular mass of ≈ 48 kDa. A hydropathy plot using the criteria of Kyte and Doolittle (1982) and homology with the rat and guinea-pig CCK-AR (Wank

et al., 1992a; de Weerth *et al.*, 1993b) identified seven regions of hydrophobic residues corresponding to putative transmembrane domains expected for members of the G-protein coupled superfamily of receptors (Dohlman *et al.*, 1987). The sequence predicts four N-linked glycosylation sites, two in the amino terminus, one in the second extracellular loop and one in the third intracellular loop. There are five potential sites for protein kinase C phosphorylation and one for protein kinase A phosphorylation (Kennelly & Krebs, 1991) all on serines in the third intracellular loop.

Receptors with seven transmembrane domains are thought to have no intrinsic enzymatic activity; instead they are coupled indirectly to their target proteins by way of membrane-delimited G proteins and second messenger molecules. This group of receptors contains at least a 100 members and represents about 80% of all known receptors (Luetje *et al.*, 1990). Despite a common overall structure, the amino acid sequences of these receptors are usually quite different. For example, the sequences of the closely related adrenergic 1 and 2 receptors are only 50% identical and the CCK-A and CCK-B receptors share 47% amino acid identity. All G protein-coupled receptors contain seven hydrophobic stretches of 20 to 25 amino acids. These form the helices that alternately are exposed either intracellularly or extracellularly. The amino acid sequences that represent the binding sites for ligands and G proteins and those that provide agonist and antagonist specificity have been identified using molecular biology techniques as mentioned above. Based on the location of the ligand binding site, GPCRs have been divided into two subclasses; receptors for glycoprotein hormones (such as thyroid-stimulating hormone) in which the binding site is thought to be in the extracellular domain (Goy, 1991) and receptors for small neurotransmitters (noradrenaline) and peptide hormones, in which the ligand binding site is likely to be within the hydrophobic transmembrane domain (Parmenter *et al.*, 1989). Recent preliminary studies on CCK-A and CCK-B receptors indicate that the agonist binding site lies in the N-terminal close to the membrane and in the second extracellular loop, respectively, (Kennedy *et al.*, 1995; Poirot & Wank, 1996).

Heterotrimeric regulatory guanine nucleotide-binding proteins (G proteins) belong to the superfamily of GTP-binding proteins or GTPases and are extremely conserved

throughout the animal kingdom, underlining their pivotal role in cellular regulations (Bourne *et al.*, 1990, 1991). They play an important role in the transduction of extracellular signals into the cell and couple receptors for light, hormones, neurotransmitters, growth factors and other extracellular signalling molecules to their intracellular effectors. Heterotrimeric G proteins consist of an α (39-45 kDa), β (35-36 kDa) and γ subunit (7-8 kDa) (Bourne *et al.*, 1990, 1991, see Figure 1.6). Each of the subunits is a product of a distinct gene. When a G coupled protein receptor (GCPR) is activated by the binding of an agonist, the molecule interacts with the heterotrimeric G protein. This interaction leads to the replacement of GDP, which is bound to the α subunit in the resting state with GTP. This nucleotide exchange induces the dissociation of the heterotrimer into the free, GTP-bound α subunit and the βγ-dimer. Both GTP bound α and βγ subunits can activate certain effectors (Tang & Gilman, 1991). This activation can be terminated by the intrinsic GTPase activity of the α subunit which cleaves the bound GTP into GDP and inorganic orthophosphate (Pi). The GDP bound α subunit now reassociates with the βγ dimer to the heterotrimer present in the resting state.

A comparison of the amino acid sequence of the human CCK-AR with rat and guinea-pig CCK-AR shows a 90 and 91% identity respectively. The human receptor is one and two amino acids shorter than the rat and guinea-pig CCK-AR, respectively. Similar to the rat and guinea-pig CCK-AR there are conserved cysteines in the first and second extracellular loops which may form a disulphide bridge required for stabilisation of the tertiary structure as demonstrated for rhodopsin (Karnik *et al.*, 1988). A cysteine in the carboxy-terminus may be a membrane anchoring palmitoylation site similar to rhodopsin (O'Dowd *et al.*, 1989).

1.3.3.2 *CCK-B receptors*

Affinity cross-linking studies of the CCK-BR using 125I-[Leu] or [N-Leu15]gastrin-(2-17),disuccinimidyl suberate in either a 60-70% pure canine gastric parietal cell preparation or a solubilised porcine gastric mucosal extract identified a 78 and a 74 kDa glycoprotein, respectively (Baldwin *et al.*, 1986; Matsumoto *et al.*, 1987).

In the rat brain, the CCK-BR was found to be ≈ 90 kDa NH_2-linked glycoprotein having a 48 kDa protein core (Wank *et al.*, 1992b). The rat CCK-BR cDNA was cloned using low stringency hybridisation from a rat pancreatic acinar carcinoma cell line (AR42J) cDNA library known to express CCK-B/gastrin receptors (Svoboda *et al.*, 1982) and shown to be identical to the cDNA cloned from the rat brain cDNA library (Wank *et al.*, 1992b). The rat CCK-BR cDNA was used to isolate the human CCK-BR cDNA from the brain and stomach (Pisegna *et al.*, 1992a). The sequencing results showed a 447 amino acid protein with a molecular mass of approximately 48.5 kDa. A hydropathy plot using the criteria of Kyte & Doolittle and homology with other G-protein coupled receptor superfamily members identified seven regions of hydrophobic residues corresponding to putative transmembrane domains expected for members of the G-protein coupled superfamily of receptors (Lambert *et al.*, 1991; Merritt *et al.*, 1986). The sequence allows for three potential N-linked glycosylation sites, all in the amino terminus. There are two potential sites for protein kinase C phosphorylation on serines in the first and third intracellular loop and three potential sites for protein kinase A phosphorylation on serines in the second intracellular loop and amino terminus and on threonine in the third intracellular loop (Kennelly & Krebs, 1991, see Figure 1.4).

Comparison of the amino acid sequence of the human CCK-BR with rat and canine CCK-B/gastrin receptor shows an ≈ 90% identity. The human receptor is five and six amino acids shorter than the rat and canine parietal CCK-B/gastrin receptor, respectively (Kopin *et al.*, 1992). Similar to the rat and canine CCK-B/gastrin receptor there are conserved cysteines in the first and second extracellular loops which may form a disulphide bridge and a cysteine in the carboxy-terminus may be a membrane anchoring palmitoylation site similar to rhodopsin (O'Dowd *et al.*, 1989).

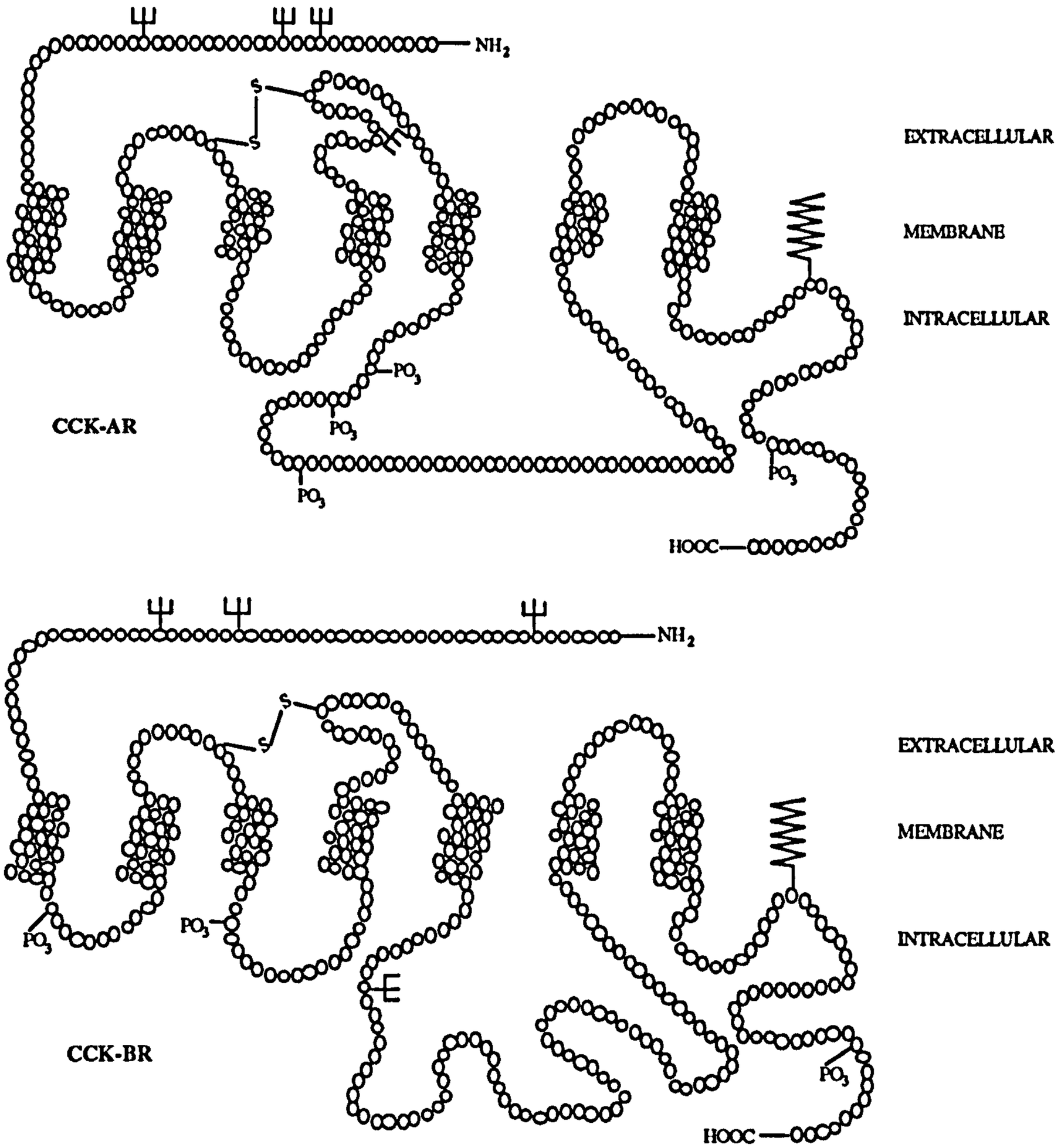


Figure 1.4 Schematic models of the rat CCK-AR and CCK-BR, showing putative transmembrane domains, consensus sites for putative N-linked glycosylation (Ψ), serine and threonine phosphorylations (PO_3) and conserved cysteines in the 1st and 2nd intracellular loops forming disulphide bridges (S-S) and a conserved cysteine in in the cytoplasmic tail possibly palmitoylated ( ), (NH_2, amino terminus; COOH, carboxy terminus).

The greatest amino acid homology between CCK receptors and other G protein-coupled receptors occurs with the gastrin releasing peptide, tachykinin and neuropeptide Y receptor families and the β_2-adrenergic and D_4 dopamine receptors. Comparison of the amino acid sequences for the rat, canine and human CCK receptor sequences to other known G-protein coupled receptors indicate that CCK receptors constitute a distinct neuropeptide receptor family within the G-protein coupled superfamily of receptors.

1.3.4 CCK receptor gene structure

The genes for the CCK-A and the CCK-B receptor have been cloned in humans (Song *et al.*, 1993). Both genes are organised in a similar manner, consisting of five exons and four introns. The receptor genes have homologous exon-intron splice sites, with exon 1 coding for the extracellular NH_2-terminal sequence, exon 2 for the sequence from the beginning of transmembrane 1 to the first part of transmembrane 3, exon 3 from transmembrane 3 to the beginning of transmembrane 5, exon 4 from transmembrane 5 to the first quarter of the third intracellular loop and exon 5 for the remainder of the receptor (see Figure 1.5).

Recently, the gastrin/CCK-B receptor was shown to be composed of five exons with an alternative splice donor site in exon 4. This results in two receptor isoforms that differ in the third intracellular cytoplasmic loop (Song *et al.*, 1993; Ito *et al.*, 1994). One receptor isoform contains 452 amino acids (long isoform) and the other lacking the pentapeptide sequence (Gly-Gly-Ala-Gly-Pro), contains 447 amino acids (short isoform). However, there is little research done on these isoforms, but it has been postulated that by the generation of receptor isoforms with differences in the cytoplasmic domain, differing signalling mechanisms could result from ligand binding.

The human CCK-AR gene has been localised to chromosome 4 (de Weerth *et al.*, 1993a) and the CCK-BR gene to chromosome 11 (Pisegna *et al.*, 1992a).

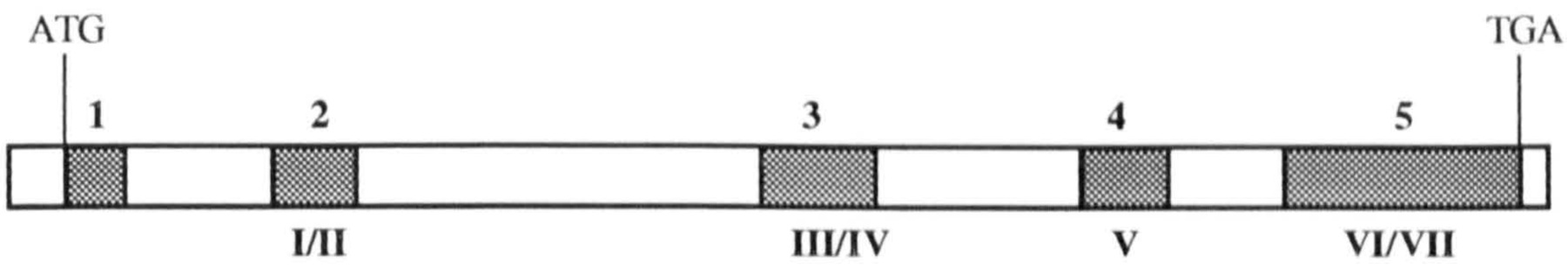


Figure 1.5 Schematic representation of genes for human CCK-AR and CCK-BR. Shown are positions of the five exons (shaded boxes) and four introns (open boxes) comprising the genes for the CCK-AR and the CCK-BR; Roman numerals refer to putative transmembrane-spanning regions encoded within each exon. ATG and TGA, putative start and stop codons, respectively.

1.3.5 Receptor signalling

1.3.5.1 *CCK-A receptors*

The signal transduction mechanisms are best characterised in pancreatic acinar cells. Occupation of cell surface membrane CCK-AR by CCK initiates coupling to pertussis toxin-insensitive guanine nucleotide binding regulatory proteins (G proteins). G protein activation and subsequent coupling to phosphoinositide specific phospholipase C (PLC) (Smrcka & Sternweiss, 1993; Taylor *et al.*, 1991) leads to the formation of inositol triphosphates (IP_3) and diacyl glycerol (DAG) (Berridge, 1987, shown in Figure 1.6). Increase in IP_3 results in release of intracellular calcium (Wakui *et al.*,

1990) and DAG activates protein kinase C (PKC). The increase in intracellular calcium appears to mediate enzyme secretion and generation of DAG is important in the sustained phase of enzyme release, which is dependent upon extracellular calcium. Enzyme secretion by CCK is mediated by both intracellular calcium and DAG and their effects on ultimate secretion are additive. Activation of PKC and intracellular calcium increase the activity of calcium-calmodulin dependent serine/threonine protein kinases and tyrosine kinases (Duan *et al.*, 1994) which lead to the phosphorylation of multiple proteins and ultimately enzyme secretion by pancreatic acinar cells. Increases in intracellular calcium induced by CCK binding to the CCK-AR are also associated with changes in monovalent cation channel activity in the plasma membrane of pancreatic acinar cells (Maruyama & Petersen, 1982). CCK stimulation causes the opening of channels that allow the influx of sodium and calcium and the efflux of potassium. Potassium efflux depolarises the membrane and results in enzyme secretion. The molecular mechanisms for the actual signalling of zymogen granule exocytosis are largely unknown. In addition to the PLC pathway, CCK at high doses can stimulate the adenylate cyclase signal transduction pathway in pancreatic acini in the presence of phosphodiesterase inhibitors.

1.3.5.2 *CCK-B receptors*

The CCK-BR in isolated canine, porcine or rabbit parietal cells, like the CCK-A receptor, couples to a pertussis toxin-insensitive G protein (Roche *et al.*, 1990), subsequently causing the activation of PLC, formation of IP_3 and DAG, release of intracellular calcium and translocation and activation of PKC (Tsunoda *et al.*, 1988, 1989).

Stimulation of the CCK-B receptor expressed in mouse and rat fibroblasts results in the phosphorylation of a number of protein species including mitogen-activated protein kinase (MAPK) and focal adhesion kinase (FAK) (Taniguchi *et al.*, 1994; Seufferlein *et al.*, 1995). The upstream mechanism by which CCK-BR interaction is coupled to the activation of MAPK has been recently examined by Seva and co-workers (1996). They showed that stimulation of the CCK-B receptor phosphorylated the adaptor protein Shc. The phosphorylated Shc subsequently associated with a complex that

includes Grb2 and the p21-Ras activator, SoS.

Compared with the CCK-AR more work has been published on the CCK-BR signalling pathways due to its trophic effects on the gastrointestinal tract.

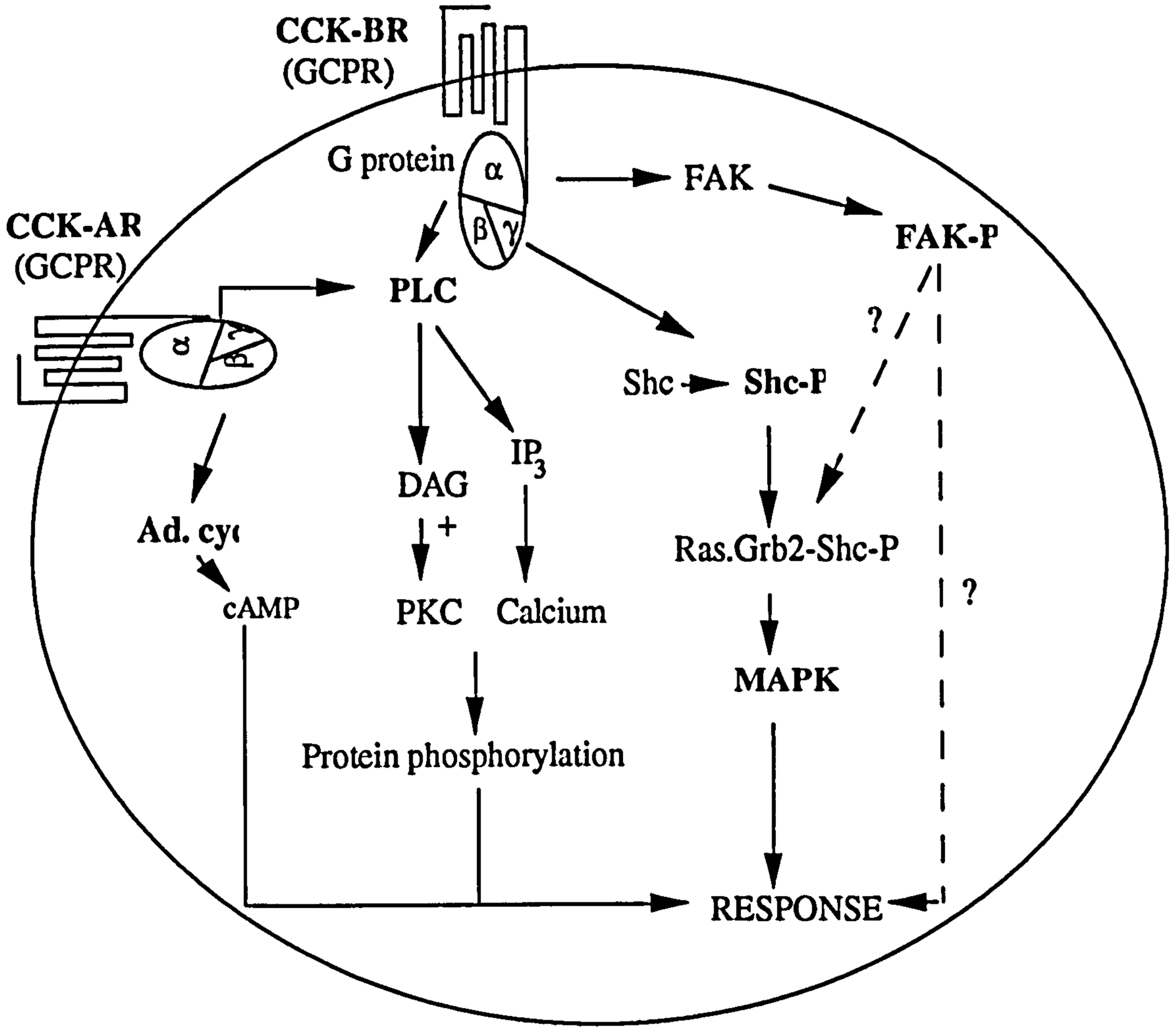


Figure 1.6 Schematic diagram showing the events following CCK-AR and CCK-BR activation. GCPR, G coupled protein receptor; PLC, phospholipase C; Ad.cyc, adenylate cyclase; cAMP, cyclic adenosine monophosphate; IP_3, inositol triphosphates; DAG, diacylglycerol; PKC, protein kinase C; FAK, focal adhesion kinase; MAPK, mitogen activated protein kinase; P, phosphorylated.

1.3.6 Novel CCK receptors

1.3.6.1 *'Gastrin/CCK-C receptors'*

The existence of an autocrine proliferative loop involving gastrin in colorectal carcinoma has resulted in the finding of a novel gastrin binding site named the 'gastrin/CCK-C receptor'. The expression of high affinity CCK-B/gastrin receptors has been reported in membranes of colorectal carcinomas and correlated to histopathological tumour staging (Upp *et al.*, 1989) but colon carcinoma cell lines generally do not possess high affinity gastrin-binding sites (Weinstock & Baldwin, 1988; Frucht *et al.*, 1992) or express CCK-A or CCK-B/gastrin receptor mRNAs (Bold *et al.*, 1994). The proliferation of colon carcinoma cell lines was inhibited by CCK receptor antagonists, proglumide and benzotript, however, the specific antagonists L-364,718 (CCK-AR) and L-365,260 (CCK-B/gastrin receptor) had no effect (Thumwood *et al.*, 1991). This novel binding site was named the 'gastrin/CCK-C receptor' by Baldwin (1994). The 'gastrin/CCK-C receptor' binds both amidated and glycine-extended forms of gastrin-17 with equal affinity and thus is a target for incompletely processed progastrins as well as mature gastrin (Baldwin, 1995).

The receptor is a 78 kDa membrane-associated protein and has been cloned from porcine gastric mucosa and found to encode a 763 amino acid protein. The protein has been shown to be a member of the hydratase/dehydrogenase family of fatty acid oxidation enzymes (Baldwin, 1995) and has been postulated to be involved in novel signalling pathways mediated by gastrin.

1.3.6.2 *Gly-G17 receptors*

A distinct gastrin receptor subtype has been found on the rat pancreatic carcinoma cell line, AR42J (Seva *et al.*, 1994). This receptor which has a high affinity for the gly-G17 requires both the N-terminal and C-terminal portions of gly-G17 for optimal binding. This receptor subtype does not recognise gastrin-17, CCK-8 or the CCK-B/gastrin receptor specific antagonists L-365,260 and PD134308. Gly-G17 has been shown to stimulate the proliferation of the rat pancreatic carcinoma cell line, AR42J (Seva *et al.*, 1994). Both high and low affinity receptor subtypes exist, which appear

to be proteins of different sizes (Seva *et al.*, 1995).

Gastrin and gly-G17 stimulated the proliferation of Swiss 3T3 cells. These cells do not express CCK-A or CCK-B receptors. The novel binding site binds amidated and glycine extended forms of gastrin with almost equal affinity and cross linking studies with ^{125}I-G2-17 showed that the molecular mass of the gastrin receptors on these cells was approximately 45 kDa (Singh *et al.*, 1995a). Whether this receptor subtype is the same as the 'gastrin/CCK-C receptor' that exists in different conformational states *eg.* glycosylation, phosphorylation, remains to be elucidated.

A truncated isoform of the human CCK-B/gastrin receptor has been recently reported (Miyake, 1995). This isoform called delta CCK-B receptor, lacks the amino terminal extracellular domain of the CCK-B/gastrin receptor. It was isolated from the human stomach and shown to have differing ligand selectivity as demonstrated by transfection into COS-7 cells. In competition studies with the antagonists L-365,260 and L-364,718, delta CCK-BR showed the properties of a classical CCK-B/gastrin receptor. However, competition studies with the agonists CCK-8 and gastrin demonstrated that delta CCK-B and CCK-B/gastrin receptor showed approximately 100 fold and 10 fold higher affinities for CCK-8 than for gastrin, respectively.

1.3.6.3 *G1/G2 receptor subtypes*

Recently evidence has been published to suggest the existence of two CCK-BR subtypes G_1 and G_2 (Harper *et al.*, 1996a; Roberts *et al.*, 1996), due to the different affinities of the selective CCK-BR antagonist L-365,260. The studies in the rat cortex and guinea pig gastric glands gave two affinity values (pKi) that suggest two receptor sites, one with a high affinity (G_2) and the other with low affinity (G_1). In the mouse cortex, rectangular hyperbolae of slope 1.0 implied the presence of a single population of binding sites (G_2). No molecular evidence has been presented to support the subtypes. It has been suggested that these receptor subtypes may be explained by: i) both glycosylation and phosphorylation of the receptors and ii) the presence of the long and short isoforms of the CCK-BR (Song *et al.*, 1993).

1.4 CCK RECEPTOR ANTAGONISTS

The process of inhibition or prevention of agonist-induced response is termed antagonism, and the chemical entities with such properties are antagonists. Receptor antagonists for gastrointestinal peptides have several common characteristics. Many antagonists are peptides that were developed by modifying the structure of an agonist by substitution of one or more amino acids or by introducing a reduced peptide bond or cyclic configuration.

In the 1980's cholecystokinin receptor antagonists were few and far between. This was mainly due to their rapid degradation by endogenous proteases, poor absorption and transport across the blood/brain barrier, low specificity and partial agonism (Freidinger, 1989). Recently, however, a number of nonpeptide receptor antagonists have been developed, many of which are highly potent.

CCK receptor antagonists can be classified into a number of groups:

1.4.1 Cyclic nucleotides

Studies of receptor antagonists for gastrointestinal peptides began with the observation that dibutyryl guanosine 3', 5' -cyclic monophosphate was a competitive antagonist of the action of CCK on pancreatic acinar cells (Peikin *et al.*, 1979). This antagonist is very weak in comparison to antagonists of CCK receptors that have since been developed. The dibutyryl group appears to be the major component for determining CCK antagonist activity (Freidinger, 1989).

1.4.2 Amino acid derivatives

Amino acid derivatives of glutamic acid have been found to possess CCK antagonistic properties. Proglumide and benzotript are more potent CCK antagonists than the cyclic nucleotide (Hahne *et al.*, 1981). However, compared with other CCK-R antagonists proglumide and benzotript are weak CCK-AR antagonists (Freidinger, 1989). They are active after oral administration (Verspohl *et al.*, 1988) and proglumide has been shown to inhibit the growth of mouse colon cancer (Beauchamp *et al.*, 1985). In

determining the structural requirements for the interaction of the amino acid derivative class of antagonists, analogues/derivatives of proglumide were developed. These include lorglumide (formerly CR 1409), loxiglumide (formerly CR1505) and KSG-504 ((S)-arginium (R)-4-[*N*-(3-methoxypropyl)-*N*-pentylcarbamoyl]-5-(2 napthylsulfonyl) pentanoate monohydrate).

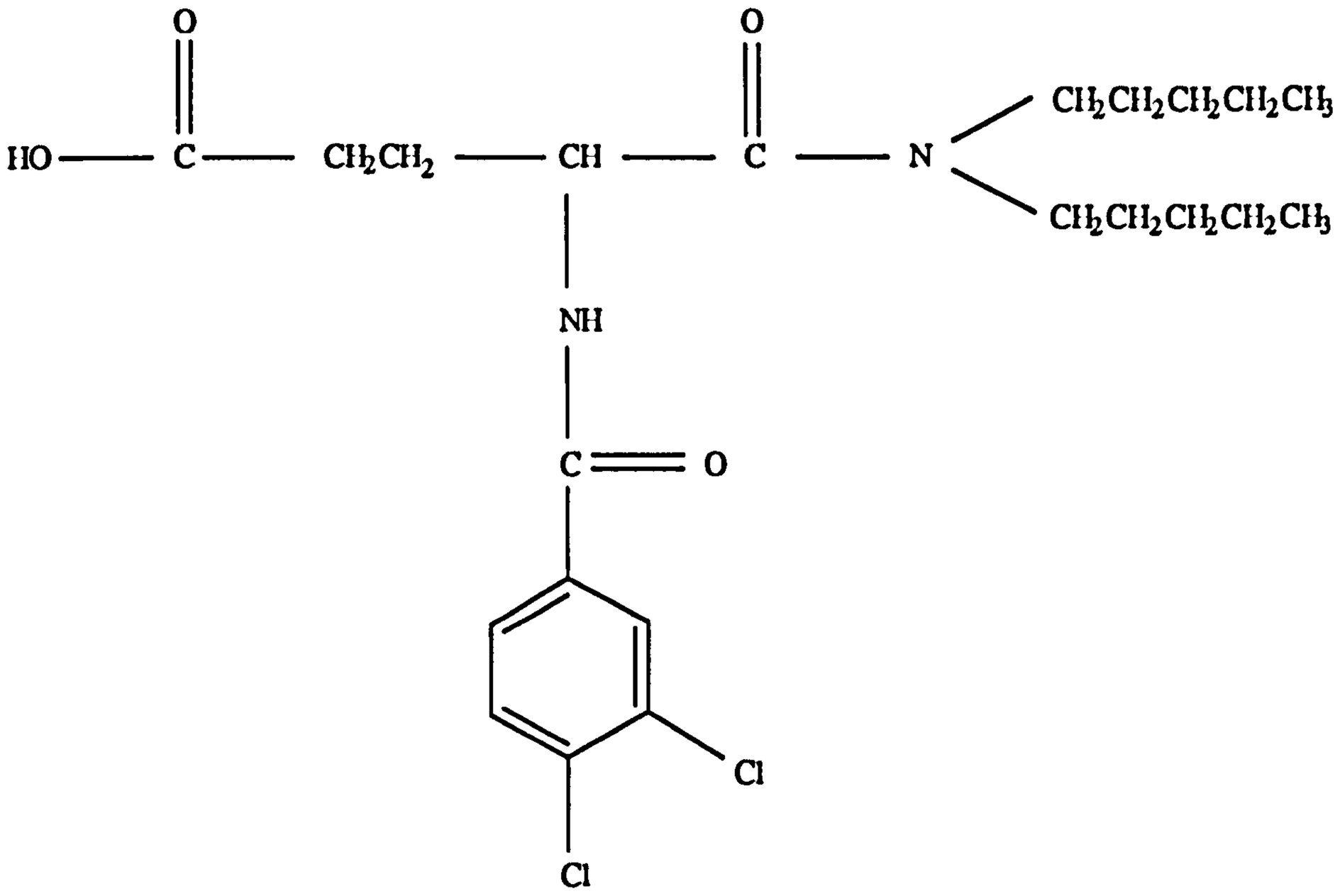


Figure 1.7 The structure of lorglumide (formerly CR 1409, DL-4-(3, 4-dichlorobenzoylamino)-5-(di-n-pentylamino)-5-oxo-pentanoic acid).

Lorglumide and loxiglumide are competitive CCK-A receptor antagonists, lorglumide being approximately 5600-fold more potent than proglumide (Freidinger, 1989). KSG-504 and lorglumide inhibit CCK-8 stimulated amylase release in the isolated rat pancreatic acini and CCK-8 stimulated exocrine pancreatic secretion in the isolated perfused rat pancreas (Kihara & Otsuki, 1995). In another study lorglumide inhibited

CCK-induced pancreatic tumour growth in rats (Douglas *et al.*, 1989).

Loxiglumide has been widely utilised in a number of pharmacological studies, including gall bladder, guinea-pig ileum and in feedback regulation of pancreatic secretion in man (Konturek *et al.*, 1989; Adler *et al.*, 1989). It also inhibits the gastrin-stimulated DNA synthesis in the rat pancreatic tumour cell line, AR42J (Seva *et al.*, 1990b).

1.4.3 Peptide and pseudopeptide analogues

JMV180, the CCK analogue, (Boc-Tyr (SO_3H)-Nle-Gly-Trp-Nle-Asp-O-CH_2-CH_2-C_6H_5), lacking the C-terminal amide function, exhibits partial agonist activity of CCK in rat pancreatic acini. It has been hypothesised that compound JMV180 interacts with both low and high affinity peripheral CCK-AR binding sites. It acts as an agonist at the high affinity binding sites and as an antagonist at low affinity binding sites (Martinez *et al.*, 1985).

Since the development of JMV180, several other analogues have been synthesised (JMV310, JMV320, JMV328 and JMV332) which exhibit high and selective affinity for CCK-B receptors in the rat and guinea pig brain (Rodriguez *et al.*, 1990). As compared with the CCK nonpeptidic compounds (Bock *et al.*, 1989) such as L-365,260, these analogues are approximately 20-100 times less potent. These peptides have however proven to be valuable in providing information about the active conformation of the CCK-B receptors.

1.4.4 Benzodiazepine derivatives

The CCK receptor antagonists in this class of compounds were synthesised after the discovery of asperlicin. The benzodiazepine, asperlicin was isolated from the fungus *Aspergillus alliaceus*. Aspercilin itself has low water solubility and poor oral bioavailability which led to the design and synthesis of more water soluble, highly potent compounds. The benzodiazepine derivative, devazepide (also known as L-364,718 and MK329) proved to be a potent, orally available CCK-A receptor antagonist (Chang & Lotti, 1986).

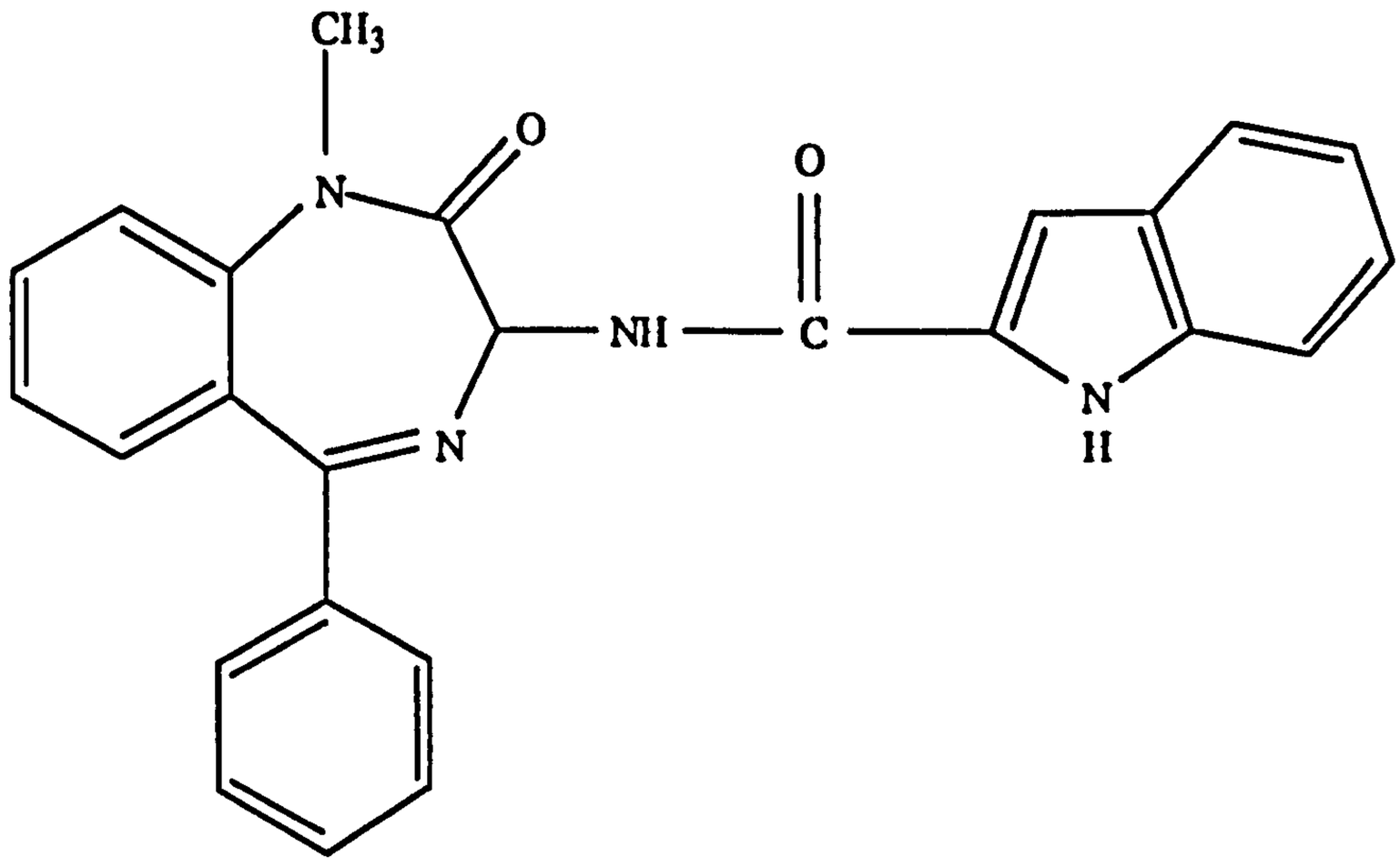


Figure 1.8 The structure of devazepide (formerly MK329 and L-364,718; 3S(-)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-1H-indole-2-carboxamide); Chang & Lotti, 1986.

Devazepide possesses potent CCK-A receptor blocking activity in various tissues. This antagonist inhibits pancreatic amylase secretion (Anderson & Dockray, 1988) with a potency 600-fold greater than CR 1409. Additional animal studies with devazepide have suggested that this agent can transiently increase food intake, enhance morphine analgesia and prevent the development of morphine-induced tolerance (Silverman *et al.*, 1987).

L-365,260, another benzodiazepine-derivative, is a potent and highly selective CCK-B receptor antagonist (Bock *et al.*, 1989; Lotti & Chang, 1989). This antagonist shows specificity for CCK-B receptors in numerous tissues including guinea-pig ileum (Botella *et al.*, 1992; Bishai *et al.*, 1993), guinea-pig gall bladder (Grider & Makhlouf, 1990) and guinea-pig stomach (Lotti & Chang, 1989). Due to the lack of water-solubility of the above antagonists, more benzodiazepine-derivatives of this form were synthesised.

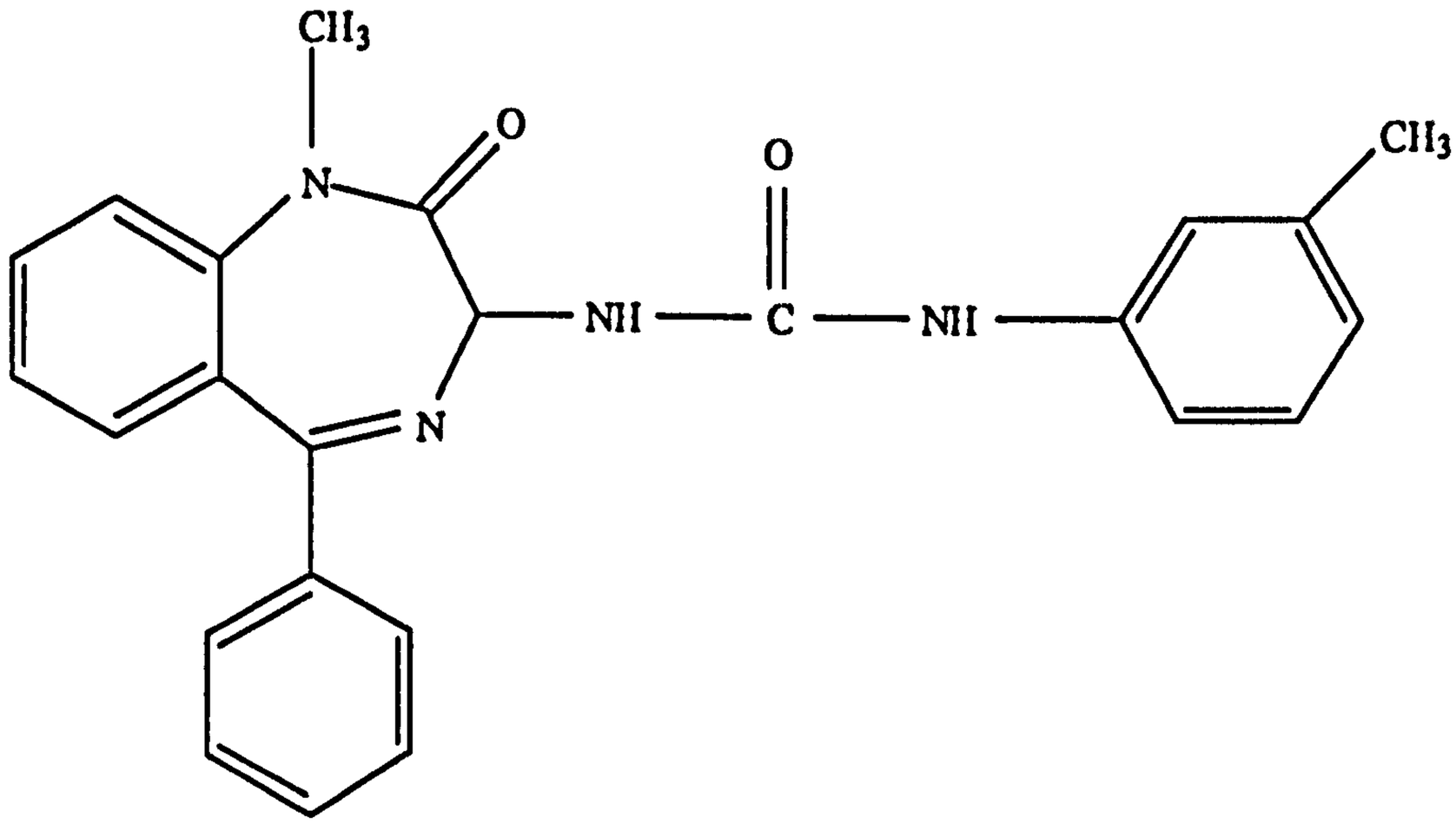


Figure 1.9 The structure of L-365,260; (3R)-(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N^1-(3-methylphenyl); Lotti & Chang, 1989.

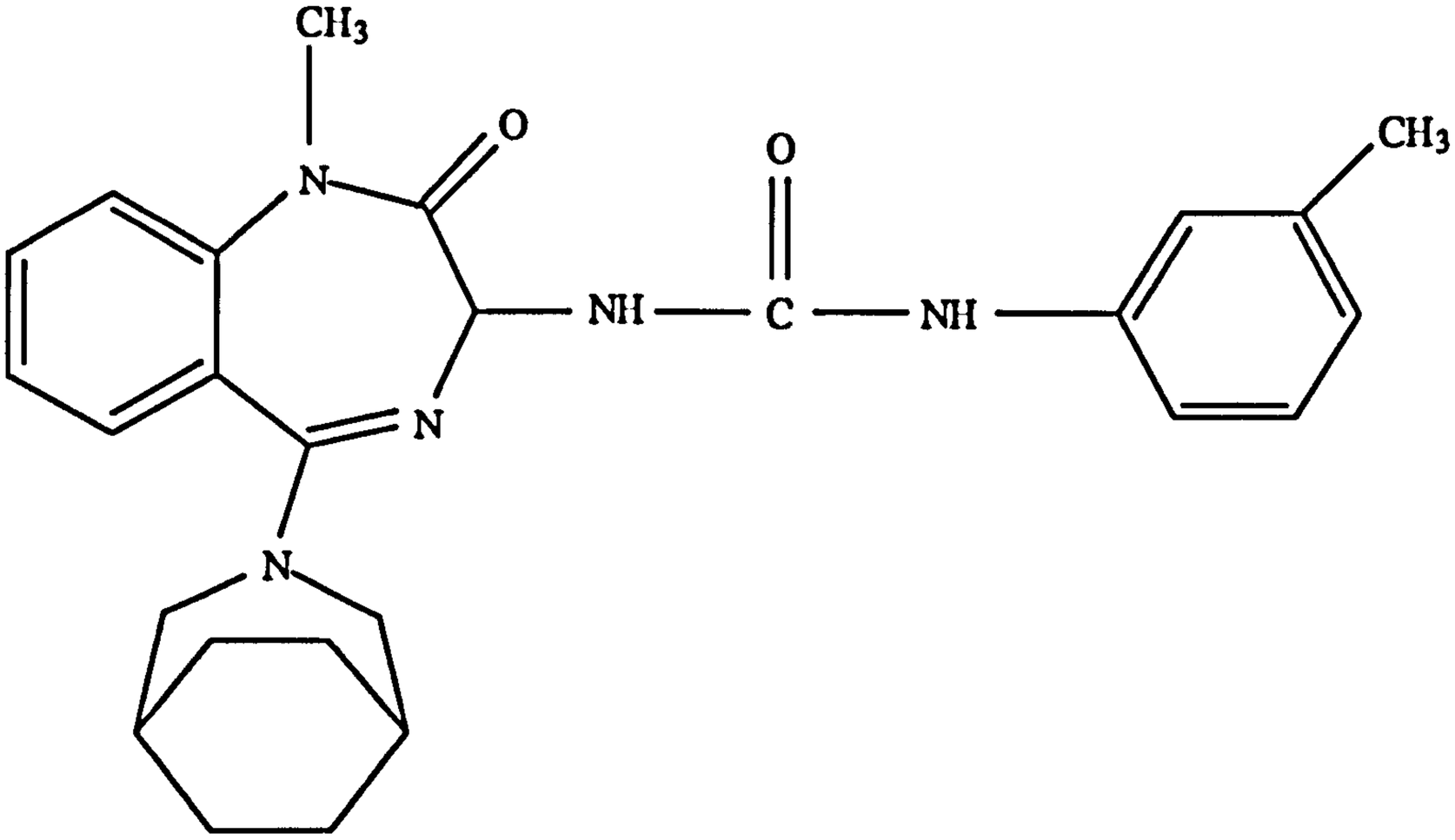
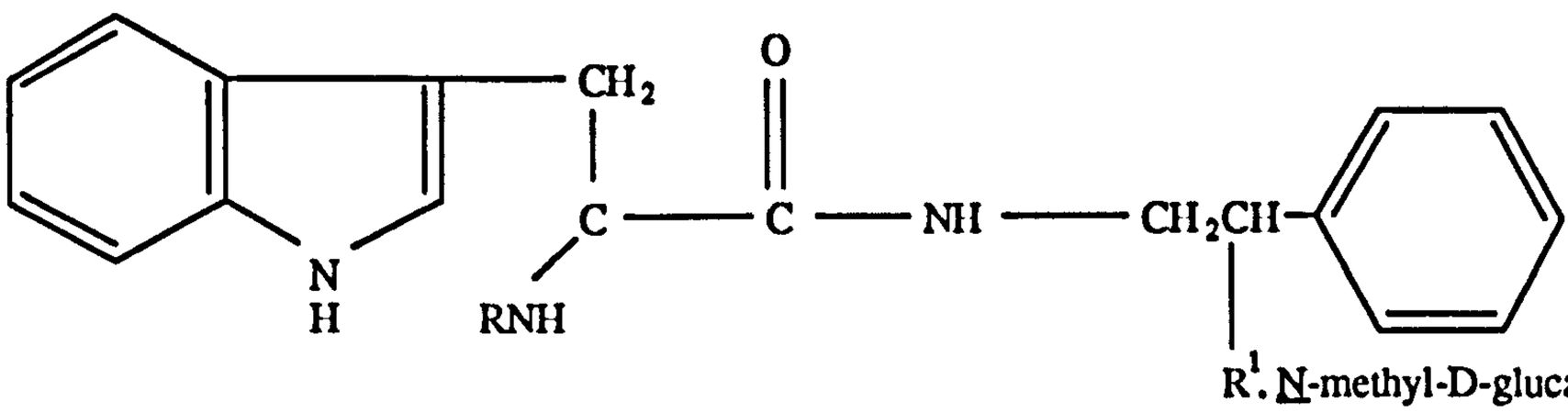


Figure 1.10 The structure of L-740,093; [N-[(3R)-5-(3-azabicyclo[3.2.2]nonan-3-yl)-2,3-dihydro-1-methyl-2-oxo-1H-1,4-benzodiazepin-3-yl]-N'-(3-methylphenyl) urea].

One exceptionally potent and water soluble CCK-B receptor antagonist, L-740,093 has a lower IC_{50} in the guinea-pig cortex (approximately 80 fold lower) than L-365,260 (Showell *et al.*, 1994). It is the most potent CCK-B receptor antagonist reported to date.

1.4.5 Peptoids

This group of compounds consists of both CCK-A and CCK-B receptor specific antagonists. Initially, these included CI-988 (formerly Cam-958 and PD134308, Hughes *et al.*, 1990) and Cam-1028 (formerly PD135158), which are potent CCK-B receptor antagonists (Woodruff & Hughes, 1991). Both antagonists are active *in vitro* and *in vivo* (Horwell *et al.*, 1991).



Name	R	R^1
CI-988	2-adamantyloxycarbonyl-	$-NHCOCH_2CH_2CO_2H$
Cam-1028	1(S)-endobornyloxycarbonyl-	$-NHCOCH_2CH_2CO_2H$

Figure 1.11 The structures of CI-988 and Cam-1028; CI-988 (formerly Cam-958, PD134308-0553;4-{[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2[[(tricyclo[3.3.1.1^{3,7}]dec-2-yloxy)carbonyl]amino]propyl]amino]-1-phenylethyl]amino}-4-oxo-[R-(R*,R*)]-butanoate N-methyl-D-glucamine.
Cam-1028;(4-{[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2[[[1.7.7 trimethylbicyclo[2.2. 1]hept-2-yloxy]carbonyl]amino]-1-phenylethyl]amino-4-oxo-1S-1α2ß[S*(S*)4α]}-butanoate N-methyl-D-glucamine); Hughes *et al.*, 1990.

More recently another group of these dipeptoid series have been synthesised with CCK-A receptor antagonistic activity. These include PD140548 and PD142896 (Singh *et al.*, 1995b). These antagonists induce potent anxiolytic-like action in rats and antagonise CCK-mediated contractions of the guinea-pig gall bladder (Singh *et al.*, 1995b) Interestingly, the enantiomers of these CCK-A receptor specific antagonists displayed reverse selectivity.

1.4.6 Indoles

Recently, JB93182 has been developed and shown to be a high affinity CCK-B/gastrin receptor antagonist with G_1 subtype selectivity (Harper *et al.*, 1996b, see Figure 1.12). Both G_1 and G_2 receptor subtypes are shown to be present in the rat cortex and guinea pig gastric glands.

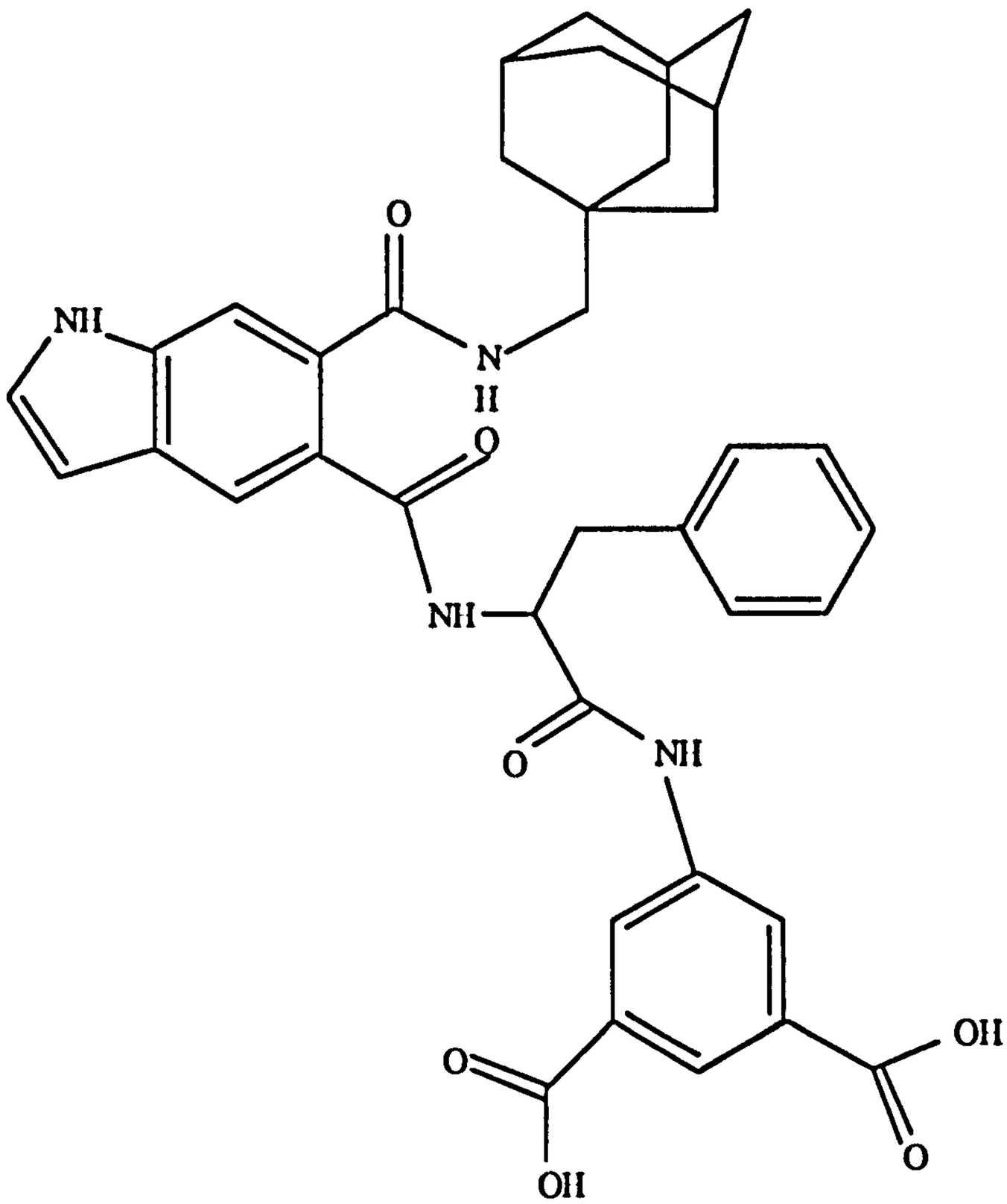


Figure 1.12 The structure of JB93182; (5[[[(1S)-[[(3,5-Dicarboxyphenyl)amino] carbonyl)-2-phenylethyl]amino]-carbonyl]-6-[[(1-adamantylmethyl)amino]carbonyl]-indole).

1.5 CANCER

Cancer is a disease, the principal characteristic of which is the heritable alteration in genetic expression of the neoplastic cell compared with its normal counterpart (Pitot, & Dragan, 1991).

Cancer has a multifactorial aetiology and is a multistep process involving initiation, promotion and tumour progression (Berenblum, 1941; Berenblum & Shubik, 1949). Chemical carcinogens, physical agents, ionising radiation, viruses and other agents have all been implicated and host factors are clearly involved (Harris, 1991; Schwab & Amler, 1990). Genetic changes have been demonstrated by virtually every possible type of mutation described, including transitions, transversions, deletions of various sizes, chromosomal rearrangements, gene amplification and insertional mutagenesis (Harris, 1991; Schwab & Amler, 1990; Gray, 1991). In a malignant neoplasm many different kinds of genetic alterations can be seen within cells of a single neoplasm. In addition such genetic alterations may evolve from simple to more complex types and numbers of mutations within the same neoplastic cell (Aldaz *et al.*, 1987; Bremner & Balmain, 1990).

Many chemical and physical carcinogens can induce one or more of a variety of mutations in cells when given chronically. Chemical carcinogens include aromatic amines (Neumann, 1986), polycyclic hydrocarbons (Pelkonen & Nebert, 1982) and methylating agents (Singer, 1986; Loeb & Preston, 1986). The main physical carcinogen is ionising radiation. Virtually all of these are capable of inducing cancer following the administration of a single or a few doses. This is found to be consistent with the fact that genetic alterations, even when induced acutely but without killing the cell, can result in cancer. However, a number of chemicals also exist that are carcinogenic only when administered in relatively high doses for extended periods of time. Many of these chemicals lack the ability to induce structural modifications directly in the DNA of the target cell.

Oncogenic viruses can cause structural defects in the DNA of affected cells. The structural organisation usually consists, at least initially, of the presence of all portions of the viral genome incorporated covalently into the host cell genome. Such viruses include papovaviruses, for example SV40, (Fareed & Davoli, 1977), adenoviruses (Branton *et al.*, 1985), and hepadnaviruses, such as hepatitis B (Slagle *et al.*, 1992). In the case of retroviruses such as Rous sarcoma, reverse transcriptase must act to make a DNA copy of the viral genome before the incorporation of the viral DNA copy into the host cell genome (Varmus, 1988). Other viruses, such as Epstein-Barr virus (Ooka, 1985) and papillomavirus (Smith & Campo, 1985) may infect cells but their genomes are not incorporated into the host cell DNA. Rather the viral genomes exist and replicate within the nuclei of the infected cells as episomes. Such an alteration may be analogous to the 'epigenetic' chemical carcinogens that stimulate cell replication but do not directly alter the structure of the host cell genome (Grasso & Hinton, 1991).

Many of the oncogenic viruses have specific genes within their own genomes whose products appear to be primarily responsible for the conversion of a normal to a neoplastic cell. These genes, termed oncogenes, act in a dominant manner and in the case of retroviral oncogenes, have counterparts within the host cell genome (Bishop, 1985; Ratner *et al.*, 1985). Because there is significant evidence to suggest that oncogenes of retroviruses evolved by mutational events from their counterparts within normal cells, the term proto-oncogene has been used to denote the cellular form of such genes.

1.5.1 The biology of carcinogenesis: Multiple stages

The initiation and progression stages of cancer involve heritable genetic changes within cells. The intermediate stage of promotion does not involve direct structural changes in the genome of the cell, but generally is characterised by an alteration in the expression of the genome of the initiated cell. Such alterations, resulting from the interaction of the genetic alterations that induced initiation and environmental factors, are termed 'promoting agents'. Unlike initiation and progression, the stage of promotion requires extended treatment with the promoting agent for the stage to be developed and to traverse into the stage of progression. These alterations in genetic expression involve a

selected enhancement of the replication of initiated cells, in the presence of the promoting agent, as well as genes (Dragan & Pitot, 1992).

1.5.1.1 *Molecular characteristics of the stage of initiation*

The stage of initiation has been studied in detail in few systems. The reason for this is the inability to identify, isolate and specifically characterise initiated cells. The experimental system in which the stage of initiation has been characterised and quantified is the rat liver (Pitot *et al.*, 1989; Moore *et al.*, 1987). In this system, the genetic alterations leading to initiation do not result in chromosomal alterations but are the result of one or more simple mutations or small deletions. Most of these initiated cells do not continue through to the subsequent stages to cancer but remain in the organism, quiescent for a lifetime. Hence, adult organisms possess numerous initiated cells in most organs that are harmless throughout life. This first stage in the development of cancer is an extremely common event that often occurs spontaneously and may be readily induced in experimental animals and in the human (Pitot & Dragan, 1991).

1.5.1.2 *The molecular biology of the stage of promotion*

The stage of promotion does not involve growth of initiated cells or molecular changes in the structure of DNA but rather changes in the expression of the genome. Thus, molecular studies of this stage are primarily concerned with the transduction of information from environmental signals to the genome with its subsequent expression. Many chemically defined promoting agents mediate their effects through receptor molecules. These receptor molecules in combination with the promoting agent as a ligand interact or bind in a non-covalent manner to specific regions of the DNA, usually upstream from specific genes. The binding of receptor-ligand complexes in turn alters the expression of the downstream gene(s). It is proposed that specific promoting agents are capable only of promoting the replication and development of specific subsets of initiated cells, presumably those whose set of genetic alterations (induced during initiation) allow optimal interaction with the promoting agent for the induction of this stage (Dragan & Pitot, 1992).

1.5.1.3 *The molecular biology of the stage of progression*

The stage of progression was originally designated by Foulds (1954) as the entire process of carcinogenesis following the stage of initiation. However, because the stage of promotion is a reversible, non-genetic process that immediately follows the stage of initiation, the original concepts of Foulds have now been modified. However at sufficiently high doses of carcinogenic agents or under specific conditions promotion does not always occur during carcinogenesis. More commonly the stage of progression develops from cells in the stage of promotion.

The characteristics of this stage include the appearance of malignant neoplasms accompanied by major genetic alterations that involve structural changes within the karyotype of the cells. Such changes include the incorporation of viral genomes or fragments thereof into the host cell genome as a result of infection. Thus, the infection of a cell with an oncogenic virus whose genome is incorporated into the host cell genome in total or in part by definition results in a by-pass of the stage of promotion. In addition progression is characterised by a continuing evolution of chromosomal abnormalities within the cell, leading to multiple stages or changes (Foulds, 1954). Cells in the stage of progression can evolve in such a way that these independent characteristics such as invasion, metastatic growth, anaplasia and the rate of growth, progress to higher and higher degrees of malignancy.

The molecular characterisation of the stage of progression as well as involving multiple molecular changes within the genome also results in the instability of its karyotype, whereas in the normal cell the structure of the genome and karyotype upon multiple cell divisions is regulated. The constant molecular shuffling is the basis also for most drug-resistance that develops in malignant neoplasms (Stark, 1986), the ability of neoplasms to escape host immune mechanisms (Goodenow *et al.*, 1985) and the abnormal expression of genes such as those normally expressed only during fetal life (Mundy, 1987). In the final analysis, the stage of progression is the one in which the physician must treat the disease in the hope of a cure. The stage of promotion has the greatest potential for preventing further development of the neoplastic process.

1.5.2 Critical molecular targets during the stages of initiation, promotion and progression

The molecular study of cancer has identified two major classes of genes that are critical molecular targets for the development of cancer. These are oncogenes (Garrett, 1986) and tumour suppressor genes (Boyd & Barrett, 1990, see Figure 1.13).

1.5.2.1 *Proto-oncogenes*

Proto-oncogenes are genes present in normal cells controlling cell growth, proliferation and differentiation. These proto-oncogenes and the oncogenes seen in tumour cells are designated by a three letter abbreviation based on the animal or tumour in which they were first identified (for example, c-Abl, cellular homologue of oncogene product from Ableson murine leukemia virus). Proto-oncogenes are capable of regulating growth by producing various protein products that form an intracellular communication network that controls cell growth. Proto-oncogenes are thus crucial to growth regulation in normal tissues. The protein products of proto-oncogenes control growth at one or more steps in the growth signalling pathway. Some proto-oncogene products include growth factors and their receptors.

1.5.2.2 *Oncogenes*

Oncogenes are genes present in human cells, that were originally identified in retroviruses. Oncogenes are very closely related to proto-oncogenes but they have mutated to produce abnormal products, or control mechanisms have altered to allow gene over-expression losing the normal constraints on their activity. Oncogene products (oncoproteins) are often closely related to normal cell growth regulators such as growth factors, growth factor receptors, signal transducers or transcription regulators.

Oncoproteins do not need external activation signals (Studzinski, 1989) for stimulation. Oncogenes are associated with carcinogenesis but are not sufficient for tumour formation. Some carcinogenic agents such as viruses can affect proto-oncogenes by stealing genes, by inducing translocations, or by inserting DNA near or within proto-oncogenes and thereby influencing their activity. Some viruses may insert their

oncogenes into host DNA, other viruses can affect tumour suppressor genes. Chemical carcinogens or ionising radiation can also produce similar effects on proto-oncogenes.

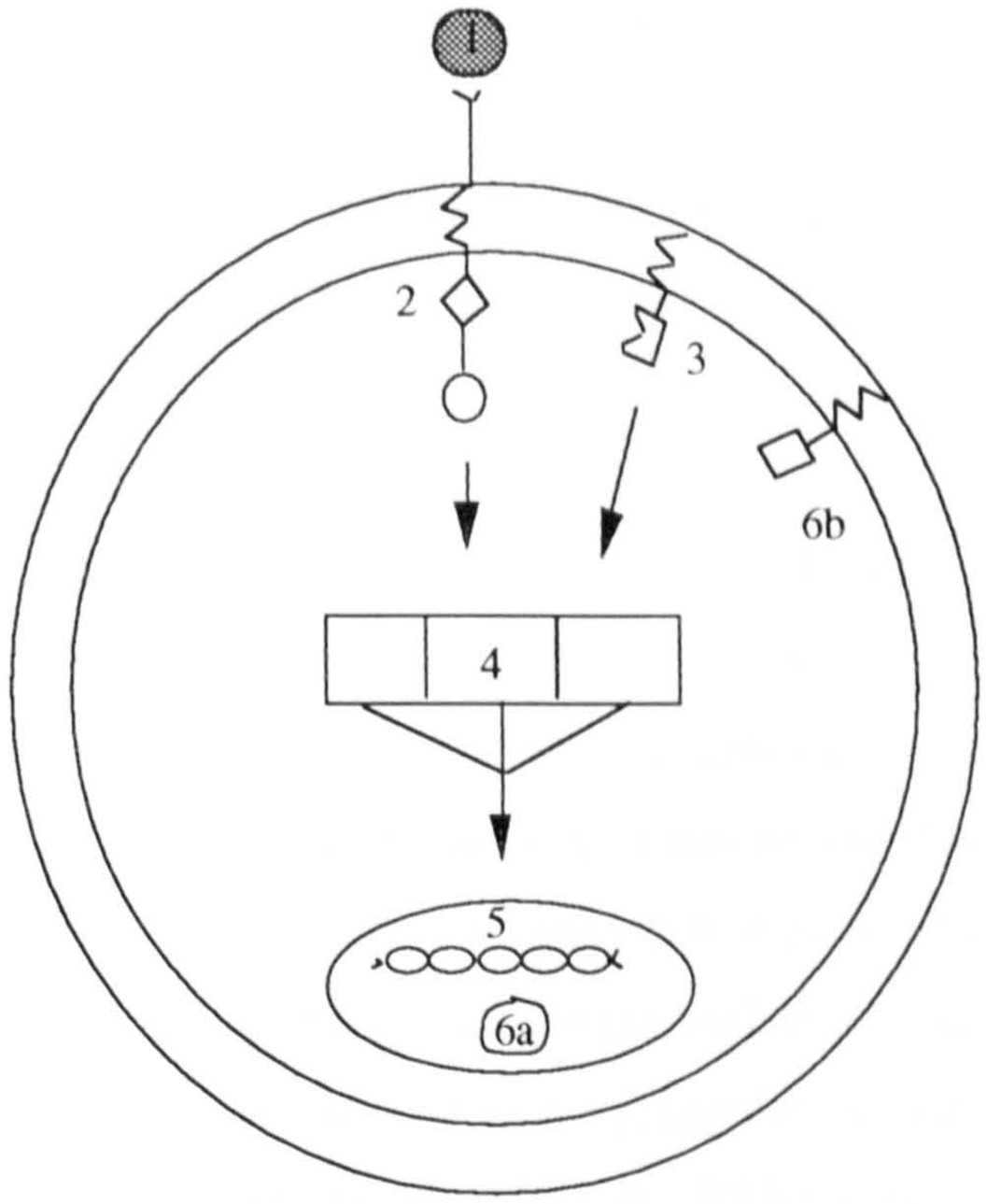
Proto-oncogenes can be activated by a number of oncogenic agents acting in one of four ways: i) Point mutations ii) Transduction and insertional mutagenesis iii) Chromosomal rearrangements and chromosome translocations iv) Amplification producing many copies of the proto-oncogene.

Each oncogene may act in a distinct way in a cell and the subsequent changes may be complementary to the alterations induced by other oncogenes, hence working in collaboration in the development of carcinogenesis (Bishop, 1985). To date more than 50 oncogenes have been identified and their associations with carcinogenesis are varied. Hence, oncogenes can lead to the uncontrolled or autonomous cell proliferation of malignant neoplasia.

1.5.2.2.1 *Oncogenes resulting in abnormal G proteins*

Gain of function mutations have been well characterised for seven transmembrane receptor G-protein α subunits (Gupta *et al.*, 1992a). The activating mutations of G-protein α subunits are similar to transforming mutations in the low molecular weight GTP binding protein ras. These include the *gsp* oncogene and the *gip*2 oncogene (Lyons *et al.*, 1990).

Smaller G proteins such as p21ras function in an analogous manner to the heterotrimeric G proteins. p21ras is an important mediator of growth factor induced differentiation and proliferation. Ras proteins belong to an extended family of GTPases (Bourne *et al.*, 1990, 1991) which includes proteins involved in protein synthesis and signal transduction (heterotrimeric G proteins). Signalling pathways from growth factor receptors, by p21ras, to several cellular effectors have been identified. p21ras is the general name for three proteins encoded by the H-*ras*, K-*ras* and N-*ras* proto-oncogenes. These proteins differ structurally at their C-termini only, and thus few, if any, functional differences between the proteins have been observed.



1 Growth factors

EGF
sis
IGF-1

2 Growth factor receptors

src
ErbB-1
met
NEU

3 Post-receptor

GTP-binding transducers

HRas
KRas
NRas
Gα

4 Cytoplasmic

raf

5 nuclear

myb
myc
fos
ski

6a Tumour suppressor genes (nuclear)

p15/p16
p21-WAF
p53
WT-1
Rb

6b Tumour suppressor genes (non-nuclear)

APC
DCC
MCC
NF1

Figure 1.13 Schematic diagram showing the various oncogenes/tumour suppressor genes involved in signal transduction pathways in the control of cellular proliferation and differentiation.

p21ras is a small GTPase that cycles between an active GTP-bound form and an inactive GDP-bound form, and functions as a molecular switch in signal transduction (Shih *et al.*, 1980). In a large number of tumours, one of the genes for p21ras is mutated resulting in a constitutive active protein, and in pancreatic cancer mutated ras genes have been found.

1.5.2.2.2 *Oncogenes acting as protein tyrosine kinases*

The protein tyrosine kinases are a family of proteins built around a highly conserved domain capable of phosphorylating protein substrates on tyrosine residues. The physiologically powerful catalytic activity of this domain has been harnessed to a wide variety of metabolic demands, from transduction of extracellular growth stimuli (Yarden & Ullrich, 1988) to responding to changes within the cell (Bauskin *et al.*, 1991). Members of this family consist of sensory, regulatory and effector domains and are known as receptor tyrosine kinases (RTKs). RTKs share a common transmembrane domain with a glycosylated N-terminal ligand binding domain, in the extracellular region, and an intracellular protein tyrosine kinase domain towards the C-terminus of the protein. A single hydrophobic transmembrane region lies between these two domains. Once the RTKs are activated they stimulate various cell signalling pathways by phosphorylation of tyrosine residues on protein substrates leading to growth and/or differentiation of cells. In tumours it has been found that these RTKs may be over-expressed (Slamon *et al.*, 1987, 1989) and/or inappropriately expressed or be expressed in a ligand-independent activated form (Janssen *et al.*, 1991).

The HER2/*NEU* RTK is a member of the epidermal growth factor (EGF) receptor family (RTK) and has been found to be over-expressed in breast and ovarian cancers (Slamon *et al.*, 1989). The src protein (a cellular homologue of an oncogene product from Rous avian sarcoma virus, pp60) is in the cytoplasmic membrane and may be involved in tumour promotion, it phosphorylates tyrosine and may affect vinculin in cell adhesion (Sefton *et al.*, 1981). Polyomavirus antigens bind strongly to src protein and such an alteration can induce cell transformation by preventing the interaction with cellular factors that normally maintain the c-src protein in a quiescent phase.

1.5.2.2.3 *Oncogenes influencing DNA*

Oncogenes, such as myb and myc produce gene products that affect DNA replication and transcription, this can result in the production of growth factors or the expression of growth factor receptors. c-*myb* induces the expression of both insulin growth factor 1 (IGF1) and IGF1 receptor mRNAs (Reiss *et al.*, 1991; Travali *et al.*, 1991). As a consequence, when over-expressed in fibroblasts, the c-*myb* product abrogates the requirement for IGF1. C-*myc* is activated in a number of neoplasms. The myc oncoprotein is termed p62 and is a regulator of cell proliferation (Travali *et al.*, 1991). All types of activation of proto-oncogenes and cellular oncogenes have been described in cells during the stage of progression. However, there is no common pattern of oncogenic activation in tumours but certain neoplasms are closely correlated with the activation of specific oncogenes by a specific mechanism (Sandberg, 1982).

1.5.2.3 *Tumour suppressor genes*

The second major set of genes fulfilling the criteria of essential targets during the process of carcinogenesis are tumour suppressor genes. Tumour suppressor genes are genes whose expression is reduced or lost in cancer cells (Knudson, 1993). This lack of expression results from mutations in the genes encoding their proteins. Thus tumour suppressor proteins act as negative growth regulators. Loss of their expression in tumour cells leads to increased cell proliferation and contributes to malignant transformation. Tumour suppressors have normal functions critical to the development of differentiated tissues. There are a number of tumour suppressor genes that can be subclassified into nuclear and non-nuclear tumour suppressors.

1.5.2.3.1 *Nuclear tumour suppressor genes*

Many of the events that culminate in growth suppression occur in the nucleus where different proteins directly interact with DNA to promote or inhibit gene transcription and the production of messenger RNA (Weinberg, 1991). These DNA binding proteins (transcriptional regulators) are intimately involved in the control of gene expression and regulate cell growth. Nuclear tumour suppressor genes may function here by modulating the expression of genes necessary for cell proliferation or differentiation. Alternatively, nuclear suppressor genes may interact with proteins

involved in regulating progression through the cell cycle. Nuclear tumour suppressor genes that alter the expression or function of cell cycle regulatory proteins disrupt the balance between cell proliferation and differentiation.

The product of one class of nuclear tumour suppressor genes is comprised of proteins intimately involved in cell cycle regulation. These include proteins that phosphorylate cell-cycle regulatory proteins, such as p15 or p16 (Serrano *et al.*, 1993; Kamb *et al.*, 1994). p15 and p16 belong to the family of cyclin-dependent kinase inhibitory proteins that function to regulate cell growth negatively through direct interactions with other proteins involved in cell cycle progression. Other cell cycle regulatory proteins, *eg.* p21-WAF/CIP, are involved in the formation of regulatory protein complexes that inhibit DNA polymerase and proliferating cell nuclear antigen (PCNA) (Li *et al.*, 1994; Waga *et al.*, 1994) resulting in cell cycle growth arrest. p21-WAF/CIP1 is also an inhibitor of cell cycle-dependent kinases like p15 and p16. Loss of expression of genes encoding these proteins would result in an increase in cell proliferation by allowing the cells to enter the cell cycle. p53, another nuclear tumour suppressor gene product (Hollstein, *et al.*, 1991), acts as a regulator of p21-WAF/CIP1 so that its loss in tumours allows cells to progress through the cell cycle and proliferate. A final group of nuclear tumour suppressor proteins function as transcriptional activators and repressors such as the retinoblastoma (Rb) and Wilm's tumour (WT-1) proteins. These proteins are transcriptional regulators and their loss leads to unchecked cell proliferation.

1.5.2.3.2 *Non-nuclear tumour suppressor genes*

This class of tumour suppressor genes encodes proteins whose locus of action resides outside the nucleus. Non-nuclear tumour suppressor proteins exert their effects through a variety of mechanisms involving signal transduction pathways, cell membrane receptors and changes in the cytoskeleton. Some of these non-nuclear tumour suppressors have been identified through positional cloning of disease genes associated with specific familial cancer syndromes.

Three colon cancer tumour suppressor genes have been identified by positional cloning. These genes include adenomatous polyposis coli (APC) (Groden *et al.*, 1991), deleted

in colorectal carcinoma (DCC) and mutated in colorectal carcinoma (MCC) which all code for cytoplasmic or membrane associated proteins. The APC protein is involved in the contact inhibition of cells, a process critical for the proper formation of tissues during differentiation and development. The other two colon cancer cytoplasmic tumour suppressor genes, DCC and MCC, are less well understood. The DCC protein is expressed at high levels in the brain and shares sequence homology with the family of cellular adhesion molecules (CAMs) (Fearon *et al.*, 1990). CAMs are thought to be involved in cell adhesion (Narayanan *et al.*, 1992) and neuronal differentiation (Lawlor & Narayanan, 1992). MCC codes for a protein with sequence similarity to the G protein-coupled muscarinic acetylcholine receptor (Joslyn *et al.*, 1991).

Neurofibromatosis 1 (NF1) gene is another tumour suppressor gene identified in the autosomal dominant disorder, neurofibromatosis 1 (Xu, *et al.*, 1990). This gene encodes for a GTPase activating protein (GAP), which inactivates the ras protein that plays a critical role in cell differentiation and proliferation. Recent studies have demonstrated that NF1 can suppress cell growth and perhaps activate cell differentiation through mechanisms unrelated to p21ras regulation (Johnson *et al.*, 1994).

As with proto-oncogenes and cellular oncogenes, mutational alterations in tumour suppressor genes that result in neoplastic transformation most likely develop in the stage of progression (Bevilacqua & Caligo, 1991). The genetic alteration of a single copy of a tumour suppressor may be a critical event for the stage of initiation with alteration of the other allele occurring in the transition from the stage of promotion to that of progression.

1.6 PANCREATIC CANCER

Pancreatic cancer accounts for only 3% of adult cancers, yet, because of the high mortality associated with this neoplasm it is one of the most common and lethal cancers in the Western world. The five year survival rate for patients with pancreatic cancer is 3% for whites and 5% for blacks (Silverberg *et al.*, 1990) and only 10-20% of the pancreatic cancers are resectable when diagnosed (Casper & Kelsen, 1995).

Cancer of the pancreatic ducts is the most common form of pancreatic cancer, accounting for over 90% of the cases (Kelly & Benjamin, 1995). Tumours of ductal origin include adenocarcinoma and cystadenocarcinoma, as well as giant cell, microglandular and adenosquamous carcinoma. A smaller number of tumours are thought to be of acinar cell origin (Warshaw & Fernandez, 1992). Islet cell (endocrine) tumours of the pancreas are rare. These tumours are not always malignant, and even when metastatic, they may be associated with long survival. In addition to epithelial tumours, sarcomas and lymphomas may be seen in the pancreas.

1.6.1 Risk Factors

The most important risk factors for pancreatic cancer are gender, age and residence. Pancreatic cancer is greater in men than women (Kelly & Benjamin, 1995) and is mainly a disease of elderly urban dwellers. 80% of persons with pancreatic cancers are between the age of 60 and 80 years old (Gordis & Gold, 1993). The recently reported increase of pancreatic cancer among women may reflect the greater life span of women (Riela *et al.*, 1992). Other risk factors (urban residence, cigarette smoking, high fat diet and chronic pancreatitis due to alcohol) that are associated with pancreatic cancer can be modified significantly and may reduce the risk of this disease. For example the relative risk for cigarette smokers compared with non-smokers ranges from 1.4 to 2.3 (Zheng *et al.*, 1993), and the age of onset of pancreatic cancer in smokers is 10 years younger than it is in non-smokers (Wynder, 1975). High fat diets have also been associated with cancer of the pancreas (Longnecker *et al.*, 1985). Chronic pancreatitis and exposure to chemicals are two other risk factors that have a strong association with

pancreatic cancer. Occupational exposure has been implicated in causing pancreatic cancer such as, β-naphthylamine and benzidine in metal, mine and sawmill plant workers (Mancuso & El-Attar, 1967). In Japan, mortality due to pancreatic cancer is nearly five times greater among chemical product workers than among the general population (Hirayama, 1989).

Patients with chronic pancreatitis of any cause have a steadily increasing risk of pancreatic cancer. At 10 years, the cumulative risk is 1.8% and at 20 years it is 4% (Lowenfels *et al.*, 1993). The major cause of chronic pancreatitis is alcohol abuse. Although the direct relationship between alcohol and pancreatic cancer is weak and inconsistent, eliminating alcohol abuse reduces the risk of chronic pancreatitis and thereby the risk of pancreatic cancer (Boyle *et al.*, 1988).

Risk factors other than alcohol that have a weak association or no association with pancreatic cancer include diabetes mellitus. Up to 80% of patients with pancreatic cancer have diabetes mellitus or impaired glucose tolerance when their cancer is diagnosed (Schwarts *et al.*, 1978). Several studies suggest an association between diabetes mellitus and pancreatic cancer, but in many patients the diabetes is an early manifestation of the pancreatic cancer rather than a risk factor for the malignancy (Gordis & Gold, 1993).

1.6.2 Clinical manifestations and physical findings

The classic presentation of a patient with pancreatic adenocarcinoma includes abdominal pain, jaundice and weight loss. Abdominal or back pain is the presenting symptom in 79% of patients (Kalser *et al.*, 1985). As with abdominal pain, weight loss of more than 10% of ideal body weight is almost universal in patients with pancreatic cancer (Perez *et al.*, 1983). Jaundice is a presenting symptom in 80-90% of patients with cancer of the head of the pancreas but in only 6-13% of patients with carcinomas of the tail of the gland (Warshaw & Swanson, 1988). Other symptoms or presentations are less common. Non-specific symptoms such as nausea, vomiting, weakness and anorexia occur in 30-36% of patients. 80% of patients with cancer of the head of the pancreas have hepatomegaly (Gullick, 1959; Howard & Jordan, 1977), and 37% have

a palpable gall bladder. In contrast patients with cancer of the body and tail of the pancreas can have abdominal tenderness and pain, but fewer than 30% have hepatomegaly and jaundice. The presenting signs and symptoms of cancers of the body or tail of the pancreas are so vague that these malignancies tend to be advanced at diagnosis.

1.6.3 Staging

A standard method is used for staging tumours in order to choose the most appropriate treatment and to compare the effectiveness of new methods. The Union Internationale Contre le Cancre (UICC) method of staging is most widely used published by the American Joint Committee for Cancer Staging.

Stage I T1-2, N0, M0: No direct tumour extension into adjacent tissue and no regional lymph node involvement.
Stage II T2, N0, M0: Direct tumour extension into adjacent tissue; no regional lymph node involvement.
Stage III T1-3, N1, M0: Regional lymph node involvement with or without direct tumour extension into adjacent tissue.
Stage IV T1-3, N0-1, M1: Distant metastatic spread.

1.6.4 Diagnosis

Early detection of pancreatic cancers greatly improves the chances of cure. When tumours are detected at an early stage, resection of the pancreas results in a longer survival time (Wanebo & Vezeridis, 1996). A single method for evaluating patients suspected of pancreatic cancer has not been standardised; however several strategies have been proposed.

1.6.5 Imaging techniques

1.6.5.1 *Plain abdominal X-rays*

If pancreatic calcifications are seen on abdominal films, a diagnosis of chronic pancreatitis can be made with 95% confidence. Primary adenocarcinomas of the

pancreas almost never calcify, but 10% of benign serous (microcystic) cystadenomas have a typical sunburst calcification in the center of the multiple microcysts that constitute the lesion (Piper *et al.*, 1962; Freeny *et al.*, 1978). In contrast mucinous (macrocystic) cystadenomas have malignant potential and may appear as a curvilinear calcification in the wall of the cyst. Loss of calcification in patients with chronic pancreatitis suggests development of pancreatic cancer, but it happens rarely (Tucker & Moore, 1963; Baltaxe & Leslie, 1967).

1.6.5.2 *Ultrasound and computed tomography (CT)*

Both ultrasound and CT are widely used to diagnose pancreatic cancer. CT is preferred as it provides better definition of the tumour and the presence of ascites or liver metastases (Kelly & Benjamin, 1995). In the absence of metastasis the CT provides information as to resectability of the cancer.

1.6.5.3 *Endoscopic retrograde cholangiopancreatography (ERCP)*

For differential diagnosis of tumours of the pancreatobiliary junction, endoscopic retrograde cholangiopancreatography (ERCP) is favoured. Ampullary and duodenal carcinomas can be biopsied at ERCP. However, chronic pancreatitis is sometimes difficult to differentiate from cancer on ERCP because fibrosis or pseudocysts may produce some of the radiological features of pancreatic cancer (Kelly & Benjamin, 1995).

1.6.5.4 *Endoscopic ultrasonography (EUS)*

The use of EUS in diagnosing pancreatic cancer has gradually increased (Rosch *et al.*, 1991; Tio *et al.*, 1990; DiMagno, *et al.*, 1982; Yasuda *et al.*, 1988). Compared with other tests EUS is more sensitive in detecting small tumours and in accurately predicting nonresectability by detecting the extension of the tumour into the vessels (Rosch *et al.*, 1992).

To complement imaging methods and simplify the diagnosis of pancreatic cancer, serum and genetic markers are being used.

1.6.6 Laboratory tests and serum markers

The laboratory tests in pancreatic cancer are non-specific. Plasma glucose, serum amylase and serum lipase may be elevated among patients with pancreatic cancer but these values are also elevated among those patients with pancreatitis. Similarly, serum bilirubin, alkaline phosphatase and aspartate aminotransferase frequently are elevated in patients who have cancer of the head of the pancreas, but these abnormalities also reflect other causes of biliary obstruction (Go *et al.*, 1981). Other laboratory findings common among patients with pancreatic cancer such as elevated lactate dehydrogenase and erythrocyte sedimentation rate and decreased serum albumin are also non-specific (Fitzgerald *et al.*, 1978).

Currently no serum markers are sensitive or specific enough to diagnose pancreatic cancer when used alone. They generally lack enough sensitivity to detect early pancreatic cancer and are not organ specific (other tumours also give positive results). Although CA19-9 (an antibody against tumour associated antigens) is 70-80% sensitive it has no value in detecting early pancreatic cancer (Frebourg *et al.*, 1988). It is useful as an indicator of prognosis or a predictor of progression or recurrence of tumour after surgery. Galactosyltransferase isoenzyme II is 67% sensitive (Podolsky *et al.*, 1981) and 98% specific and the serum testosterone to dihydrotestosterone ratio in male patients is also 67% sensitive and 98% specific, but these tests are not useful in detecting early pancreatic cancer (Warshaw & Swanson, 1988). Leukocyte inhibition is useful in detecting early stage I pancreatic cancers, but is not sensitive enough for detecting stage II or III cancers. Four of five patients with stage I but only 5 out of 16 with stage III pancreatic cancer had a positive test (Meduri *et al.*, 1989).

At present, markers include K-ras mutations and plasma islet amyloid polypeptide (IAPP). K-*ras* gene mutations occur in 90% of cases of pancreatic ductal adenocarcinoma (Almoguera *et al.*, 1988) and the site of mutation is restricted to codon 12 of the K-*ras* gene. The diagnostic and clinical value of its detection in pancreatic juice, blood or stool is under intensive investigation.

IAPP is secreted by the islet cells and reduces insulin sensitivity. It is elevated in patients with pancreatic cancer who have diabetes (Permert *et al.*, 1994). Although IAPP levels contribute to diabetes, IAPP may be an ideal marker to detect early asymptomatic pancreatic cancer in the subset of 60-70% of patients with pancreatic cancer in whom glucose intolerance is secondary to the cancer.

On the basis of the literature, in general, clinicians primarily perform an abdominal ultrasound to diagnose pancreatic cancer and follow it by an ERCP. Tumour markers are commonly used but pancreatic function tests rarely so. Current data suggest that CT is the best method to diagnose and stage pancreatic cancer and that EUS should be the next test to stage the tumour. In the future EUS may become the single best test to diagnose and stage the tumour simultaneously (Kinjo & DiMagno, 1995).

1.6.7 Treatment of pancreatic cancer

Treatment of patients with advanced pancreatic cancer and evaluation of new agents for this disease present a major problem. Patients with metastatic disease are frequently debilitated at diagnosis. Evaluation of therapeutic agents has been hampered by limitations of the diagnostic tests available.

1.6.7.1 *Surgical management and adjuvant therapy*

Although surgical resection provides the only realistic chance for cure, surgery is only possible in a small number of patients. In general of every 100 patients that are surgically explored for pancreatic cancer that is presumed to be resectable, resection is only possible in 25-30%. Pancreatoduodenectomy (the Whipple procedure) is the most widely accepted operation for the resection of pancreatic cancer. Five year survival of patients with resected pancreatic carcinoma is only 20% (Geer & Brennan, 1993). Approximately 30-40% of patients explored with intent to resect are found to have metastases, usually to the liver at the time of laparotomy and another 30-40% of patients have locally advanced disease. Surgical resection for these two groups of patients is not feasible.

1.6.7.2 *Radiation therapy*

Radiation therapy has been used in the management of pancreatic cancer both as an adjuvant following resection and for the treatment of locally advanced, unresectable tumours. The limitations of this treatment modality are due to the anatomic relations to the pancreas that prohibit the delivery of tumouricidal doses of radiation. The concomitant administration of 5-fluorouracil as a radiation sensitiser appears to enhance the effect of radiation and improve survival (Kalser & Ellenberg, 1985). Although there is not much data evaluating the effect of radiotherapy on unresectable pancreatic cancer, it appears that the combination of radiation with 5-fluorouracil enhances local control (Whitington *et al.*, 1984). The limitations of external beam radiation prompted the use of intraoperative radiation therapy (IORT), which is capable of delivering high doses of radiation to the pancreas without radiation injury to the surrounding structures (Dobelbower *et al.*, 1991). Combination of IORT with external beam radiation results in a good local control (Shipley *et al.*, 1984). Overall the data available in the literature indicate that radiation therapy is unsatisfactory both as an adjuvant and in the management of locally advanced disease. However, radiation may be a recommended palliative method providing an improved quality of survival by relieving pain (Shipley *et al.*, 1984).

1.6.7.3 *Chemotherapy*

Chemotherapy is of limited value in the palliation of pancreatic cancer. Single agent chemotherapy with 5-fluorouracil has given an increase in survival of 26% (O'Connel, 1985). A similar response was observed with mitomycin C. 8-10% increase in survival was seen with doxorubicin, streptozotocin and ifosfamide (as reviewed in O'Connel, 1985). Combination chemotherapy failed to show improved responses compared to single agent treatment

Combination of chemotherapy with radiotherapy has been shown to increase the survival of the patient compared with chemotherapy alone (Moertel *et al.*, 1981). However, prognosis remains poor for patients with pancreatic cancer who cannot be surgically resected.

1.7 GROWTH FACTORS AND ONCOGENES IN PANCREATIC CANCER

It is widely recognised that alterations in the functions of growth factors and their receptors and/or their aberrant expression may lead to abnormal cell growth progressing to neoplasia (Aaronson, 1991).

One characteristic includes the diminished or complete lack of requirement for serum or specific growth factors. Neoplastic cells in culture are presumed to have autonomous activating pathways in their growth system, namely: i) autonomous production of growth factors; ii) synthesis of an altered receptor for growth factors; and iii) activation of post-receptor pathway (Sporn & Todaro, 1980; Sporn & Roberts, 1985; Goustin *et al.*, 1986).

The loss of inhibitory influences (tumour suppressor genes) and the upregulation of stimulatory mechanisms of cell proliferation (growth factors, growth factor receptors and oncogenes) are important mechanisms in the pathogenesis of pancreatic cancer.

1.7.1 Pancreatic cancer and growth factors and growth factor receptors

1.7.1.1 *Hepatocyte growth factor receptor (MET)*

The c-met proto-oncogene encodes a transmembrane tyrosine kinase receptor (MET) that has the capacity to modulate cell proliferation and differentiation (Cooper *et al.*, 1984). It is activated by the hepatocyte growth factor (HGF). HGF is a 105 kDa protein that is mitogenic toward hepatocytes, endothelial and epithelial cells (Montesano *et al.*, 1991; Zarnegar *et al.*, 1990). The HGF action is dependent on its binding to MET, the HGF receptor.

MET has been shown to be overexpressed in pancreatic cancer (Di Renzo *et al.*, 1995) and the overexpression is associated with increased levels of HGF mRNA (Ebert *et al.*,

1994). However, the importance of this upregulation is not yet determined but it may be involved in motility or invasiveness of pancreatic tumour cells (Oikawa *et al.*, 1995).

1.7.1.2 *Fibroblast growth factor (FGF)*

Members of the fibroblast growth factor (FGF or heparin binding growth factor) family are potent mitogens for a wide variety of mesodermal and neuroectodermal cells and have been isolated from a variety of tissue and cell sources including tumour cells (Thomas & Gimenetz-Gallego, 1986; Gospodarowicz *et al.*, 1987). This family of peptides currently includes acidic FGF (aFGF), basic FGF (bFGF), keratinocyte growth factor, FGF-4 (Kaposi FGF), FGF-5, FGF-6, the gene product of *int*-2, androgen-induced growth factor and FGF-9. FGFs promote angiogenesis and chemotaxis and participate in the regulation of cellular differentiation and tissue repair.

aFGF and bFGF have been shown to be overexpressed in pancreatic tumours and this overexpression is associated with a more advanced tumour stage (Yamanaka *et al.*, 1993a). To date five distinct high affinity FGF receptors, designated as FGF-1, -2, -3, -4, and -5 (the latter is also known as *flg*-2) have been cloned and sequenced (Jaye *et al.*, 1992). These receptors are tyrosine kinase receptors and the FGFR-1 type is overexpressed in pancreatic cancers (Kobrin *et al.*, 1993). As with the FGFs the overexpression of the FGFR correlates with the extent of pancreatic tumour malignancy (Ohta *et al.*, 1995), suggesting a role for autocrine stimulation in these tumours.

1.7.1.3 *Epidermal growth factor receptor (EGFR)*

The epidermal growth factor receptor (EGFR or HER) binds epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), Shope fibroma virus growth factor, myxoma virus growth factor and amphiregulin (Soyab *et al.*, 1988). TGF-α is the most extensively studied of the EGF-like molecules. The EGFR is a 170 kDa protein that possesses tyrosine kinase activity. This receptor is closely related to three additional receptors, termed, c-*erb*B2 (HER2), c-*erb*B3 (HER3) and c-*erb*B4 (HER4) (Prigent & Lemoine, 1992; Plowman *et al.*, 1993). HER exhibits a strong sequence homology with the oncogenic protein product of the avian erythroblastosis virus v-*erb*-B gene (Downward *et al.*, 1984).

Overexpression of the EGFR is associated with the malignant phenotype (Libermann *et al.*, 1984; Ro *et al*., 1988) and with the enhanced ability of certain tumours to invade normal tissues and metastasise (Sainsbury *et al.*, 1985; Neal *et al.*, 1985). Overexpression of either EGF or TGF-α also leads to abnormal cell growth and acquisition of features that are characteristic of the transformed phenotype (Watanabe *et al.*, 1987; Heidaran *et al.*, 1990). Pancreatic cancer and pancreatic cancer cell lines have been shown to overexpress the EGFR (Korc *et al.*, 1986; Lemoine *et al.*, 1992a) and EGF and /or TGF-α (Barton *et al.*, 1991a) indicating that autocrine and paracrine mechanisms of this receptor-ligand system play a crucial role in the pathogenesis of pancreatic cancer (Korc *et al.*, 1992; Yamanaka *et al.*, 1993b).

1.7.1.4 *c-erbB-2*

The c-*erb*B2 gene encodes a transmembrane receptor with a molecular weight of 185 kDa. This receptor is activated through binding of specific ligands such as *neu* differentiation factor (NDF), glial growth factors and heregulin. c-*erb*B2 is shown to be overexpressed in 45% of pancreatic cancers (Yamanaka *et al*., 1993c). In contrast with the EGFR, the presence of c-*erb*B2 is not associated with advanced tumour stage or worse postoperative survival periods.

The third member of the EGFR related family of growth factor receptors is c-*erb*B3 (Kraus *et al*., 1989). Analysis of c-*erb*B3 mRNA expression indicates that pancreatic cancer cells also overexpress this growth factor receptor and that this overexpression is associated with advance tumour stages and a significantly post-operative survival (Friess *et al.*, 1994). Although the ligand that activates c-*erb*B3 has not yet been identified the above findings suggest that the overexpression of the EGF receptor and/or c-*erb*B3 seems to play an important role in pancreatic cancer progression and patient prognosis (Friess *et al.*, 1994).

1.7.1.5 *Insulin-like growth factor (IGF)*

Insulin like growth factor-I is a polypeptide that exhibits structural homology to proinsulin and exerts growth-promoting and metabolic effects (Froesch *et al.*, 1985).

Its actions are dependent on the presence of specific cell surface receptors, the insulin like growth factor-I receptor (IGF-IR). This receptor is a tyrosine kinase receptor, which has a low affinity for insulin and a high affinity for IGF-I. IGF-I functions as an autocrine and a paracrine growth factor in a variety of mesenchymal and epithelial tumours (Macaulay, 1992; Daughaday, 1990) and is essential for entry into the S phase of the cell cycle (DNA synthesis) (Baserga, 1995).

Pancreatic tumours have been shown to overexpress mRNA for both IGF-I and IGF-IR. It has been suggested that IGF-I may play a role in the aberrant autocrine and paracrine activation of IGF-IR in pancreatic cancer (Bergmann *et al.*, 1995).

1.7.1.6 *Transforming growth factor beta (TGF-β)*

The transforming growth factor beta gene superfamily consists of numerous regulatory polypeptides that include several TGF-β isoforms as well as activins and inhibins (Massague, 1990). Mammalian cells express TGF-β1, TGF-β2 and TGF-β3 which are isoforms of TGF-β. The TGF-βs are multifunctional polypeptides that influence cell growth and differentiation, adhesion, migration, angiogenesis, extracellular matrix formation and immune functions (Massague, 1990). The effects of TGF-βs on cell proliferation *in vitro* may be either positive or negative depending on the cell type and culture condition, in most cells of epithelial origin TGF-βs act as a potent growth inhibitors (Baldwin & Korc, 1993). All three isoforms are over-expressed in human pancreatic cancer (Friess *et al.*, 1993a). This enhanced expression of TGF-β isoforms is correlated with decreased survival. The expression of TGF-β receptors, which are divided into three subgroups, shows an overexpression of TGF-β receptor II in pancreatic cancer while expression of TGF-β III is not altered compared to normal pancreatic tissue (Friess *et al.*, 1993b).

1.7.1.7 *Somatostatin*

Somatostatin is a tetradecapeptide that participates in a variety of biological processes including inhibition of hormonal secretion and cell proliferation (Lewin, 1992). These properties are used for the treatment with stable somatostatin analogues of a number of

hormone producing tumours such as gastroeneteropancreatic cancers (Lamberts *et al.*, 1991). The actions of the peptide are mediated by specific somatostatin receptors of which five have been cloned and referred to as SSTR1-SSTR5 (Bell & Reisine, 1993). Only SSTR2 and SSTR5 mediate the antiproliferative effect of somatostatin analogues octreotide and vapreotide (Buscail *et al.*, 1994). Recently it has been shown that there is a loss of the SSTR2 in pancreatic cancers (Buscail *et al.*, 1996) and that the loss of this receptor type could represent a growth advantage in these tumours and provide an explanation for the lack of therapeutic effect of somatostatin analogues in such adenocarcinomas.

1.7.1.8 *Bombesin and gastrin-releasing peptide*

Bombesin and its mammalian counterpart, gastrin-releasing peptide (GRP), are hormonal peptides which can exert diverse physiological or pharmacological actions in various systems (Spindel, 1986). In the gastrointestinal system, bombesin and GRP stimulate gastric and pancreatic secretions, enhance the release of several gastrointestinal hormones and promote the growth of the exocrine pancreas. Bombesin and GRP can function as autocrine or paracrine growth factors and stimulate the growth of normal and malignant cells including gastric cancer cells (Yano *et al.*, 1992) and CAPAN, a human pancreatic tumour cell-line (Avis *et al.*, 1988). Bombesin/GRP antagonist (RC-3095) has been shown to suppress the growth of nitrosamine-induced pancreatic cancers in hamsters (Szepeshazi *et al.*, 1991) and inhibit the growth of human pancreatic cancer cells *in vivo* and *in vitro* (Qin *et al.*, 1994).

1.7.1.9 *pS2*

pS2 is a 60 amino acid, cysteine rich, secretory polypeptide which shows some structural similarity to small growth factor-like peptides such as IGF-I (Jakowlew *et al.*, 1984). This protein belongs to a family of trefoil or three looped clover shaped structural polypeptides. These proteins are found throughout the gastrointestinal tract, particularly in association with mucosal inflammation where its primary function is thought to be as a growth factor (Wright, 1993). pS2 expression has been found in breast and colon cancers. pS2 has been shown to be expressed in pancreatic cancers but not in the normal pancreas (Welter *et al.*, 1992; Collier *et al.*, 1995). This finding is

recent and the mode of action, nature of its receptor are unknown. However, its role as a secretory peptide is thought to be mediated by an autocrine or paracrine mechanism (Collier *et al*., 1995).

1.7.1.10 *Annexin*

The protein tyrosine kinase substrate, annexin II, has been implicated in the process of DNA replication and cell proliferation. Annexin II is phosphorylated by pp60 src kinase (Gerke & Weber, 1984), protein kinase C (Gould *et al.*, 1986) and growth factor receptor kinases (Brambilla *et al.*, 1991). Annexin II is a member of the annexin family of proteins that are characterised by their ability to bind phospholipids in a calcium-dependent manner (Glenney, 1986). Annexin II stimulates DNA polymerase alpha (Jindal *et al*., 1991) and has been postulated to be involved in mitogenic signal transduction (Fava & Cohen, 1984). In recent years, elevated expression of annexin II has been demonstrated in carcinogen-induced Syrian hamster pancreatic adenocarcinoma (Kumble *et al.*, 1992), human hepatocellular carcinoma (Frohlich *et al.*, 1990) and in a multidrug resistant, small cell, lung cancer cell line (Cole *et al.*, 1992). Expression studies show annexin II expression is deregulated in pancreatic cancers resulting in an overexpression of annexin II mRNA and protein (Vishwanatha *et al.*, 1993). The literature documenting these findings is shown in Table 1.1.

1.7.2 Pancreatic cancer and oncogenes

1.7.2.1 *Ras*

Among the most frequent genetic changes in human pancreatic cancer are point mutations of the Kirsten-*ras* (rous sarcoma virus) proto-oncogene (K-*ras*). These have been identified in tumour needle biopsies and aspirations in 85-95% of pancreatic adenocarcinomas (Grunewald *et al.*, 1989; Lemoine *et al.*, 1992b; Smit *et al.*, 1988). K-ras mutations occur in pancreatic cancer almost exclusively on codon 12 (Almoguera *et al.*, 1988) which is located in the short arm of chromosome 21.

p21ras is the general name for three proteins encoded by the H-*ras*, K-*ras* and N-*ras* proto-oncogenes. p21ras is an important mediator of growth factor induced

differentiation and proliferation. Ras proteins belong to an extended family of GTPases (Bourne *et al.*, 1990; 1991) which includes proteins involved in protein synthesis (the elongation and initiation factors) and signal transduction (heterotrimeric G proteins). Signalling pathways from growth factor receptors, *via* p21ras, to several cellular effectors have been identified. H-*ras*, K-*ras* and N-*ras* proteins differ structurally at their C-termini only, and few, if any, functional differences between the proteins have been observed. p21ras is a small GTPase that cycles between an active GTP-bound form and an inactive GDP-bound form, and functions as a molecular switch in signal transduction.

Early functional studies using ras neutralising antibodies showed that in a variety of cell types, p21ras is required for growth factor induced DNA synthesis and gene expression (Stacey & Kung, 1984), as well as for the induction of differentiation. A second approach to studying the function of p21ras has been the use of dominant negative mutants of p21ras (Feig & Cooper, 1988a, 1988b). One commonly used interfering mutant is $p21ras^{asn17}$. This mutant displays a reduced affinity for GTP, but normal affinity for GDP, and interferes in the GDP-GTP cycling of normal p21ras. Introduction of this mutant also inhibits ligand-induced proliferation and differentiation in various cell types. All these results point to a function of p21ras in growth factor receptor-mediated signal transduction.

1.7.2.1.1 *Growth factor receptors activate p21ras*

There is a large amount of literature showing the involvement of ras in receptor tyrosine kinase activation, *eg*, epidermal growth factor receptor (Gibbs *et al.*, 1990). Most if not all receptor tyrosine kinases have been shown to activate p21ras. Ligands that activate (non-receptor tyrosine kinases) indirectly or certain members of the seven transmembrane receptor families also induce the activation of p21ras (Crespo *et al.*, 1994).

1.7.2.1.2 *Mechanism of p21ras activation*

Two rate-limiting steps can be distinguished in the cyclic regulation of the nucleotide

content of p21ras (Bos, 1995). First the dissociation of GDP followed by the binding of GTP and secondly, the hydrolysis of p21ras-bound GTP. p21ras exhibits a slow intrinsic guanine nucleotide exchange and a slow intrinsic GTPase activity. However, guanine nucleotide exchange proteins (GNEFs *eg.* Grb2/SOS) regulate rapid GDP/GTP exchange, and GTPase activating proteins (GAPs *eg.* p120GAP, neurofibromin) are present for the stimulation of GTP hydrolysis. p21ras can be activated by one of two mechanisms: an increase in guanine nucleotide exchange activity and/or an inhibition of GAPs.

1.7.2.1.2.1 Nucleotide binding

Rasp21 binds GTP with high affinity (Hoshino *et al.*, 1987; John *et al.*, 1990). Three sequence motifs important for nucleotide interaction have been determined which are conserved between different guanosine nucleotide-binding proteins (Bourne *et al.*, 1991). The first, GXXGXGKS (amino acids 10-17 in p21) is involved in the binding to the α- and β- phosphates; in the second, DXXG (57-60 in p21) the aspartate binds the magnesium and the glycine binds the γ-phosphate when the GTP is bound; and the third, NKXD (116-119 in p21) is important for binding to the guanine ring. In p21, residues 144-146 are also important for interaction with the nucleotide. Single mutations of residues, 10, 13, 15, 16, 83, 116, 117, 118, 119, 144 and 146 have been found to lower GTP binding (usually by 1-3 orders of magnitude), in most instances by lowering the dissociation rate constant (Feig & Cooper, 1988b; Sigal *et al.*, 1986; Walter *et al.*, 1986; Clanton *et al.*, 1987; Feig *et al.*, 1986; Der *et al.*, 1986a; Clanton *et al.*, 1986).

1.7.2.1.2.2 Mechanism of GTP hydrolysis

The ras GTPase activity results in the slow hydrolysis of bound GTP (Sweet *et al.*, 1984; Gibbs *et al.*, 1984a; Manne *et al.*, 1985), leaving the protein complexed with GDP. Mutation of residue 12 or 61 particularly effects this intrinsic rate which suggests that these amino acids are important for the p21 conformation mediating GTP hydrolysis (Der *et al.*, 1986b; Sweet *et al.*, 1984; Gibbs *et al.*, 1984a; McGrath *et al.*, 1984).

As measured by various investigators, the rate of hydrolysis gives a GTP p21 half-life of 1-5 hours for normal p21 with that of activated versions being 3-9 times longer (John *et al.*, 1988; Sweet *et al.*, 1984; Gibbs *et al.*, 1984a; Lacal *et al.*, 1986). The "catalytic" domain of p21 is generally considered to be the conserved 164 N-terminal amino acids (Gideon *et al.*, 1992).

1.7.2.1.2.3 Intrinsic exchange

The intracellular GTP concentration is much greater than that of GDP, thus, the GTP is bound to the native p21 protein as it is synthesised and acquires its tertiary structure. The intrinsic rate of dissociation is very low, approximately 10^{-5} moles per second dissociating per mole of complex (John *et al.*, 1990).

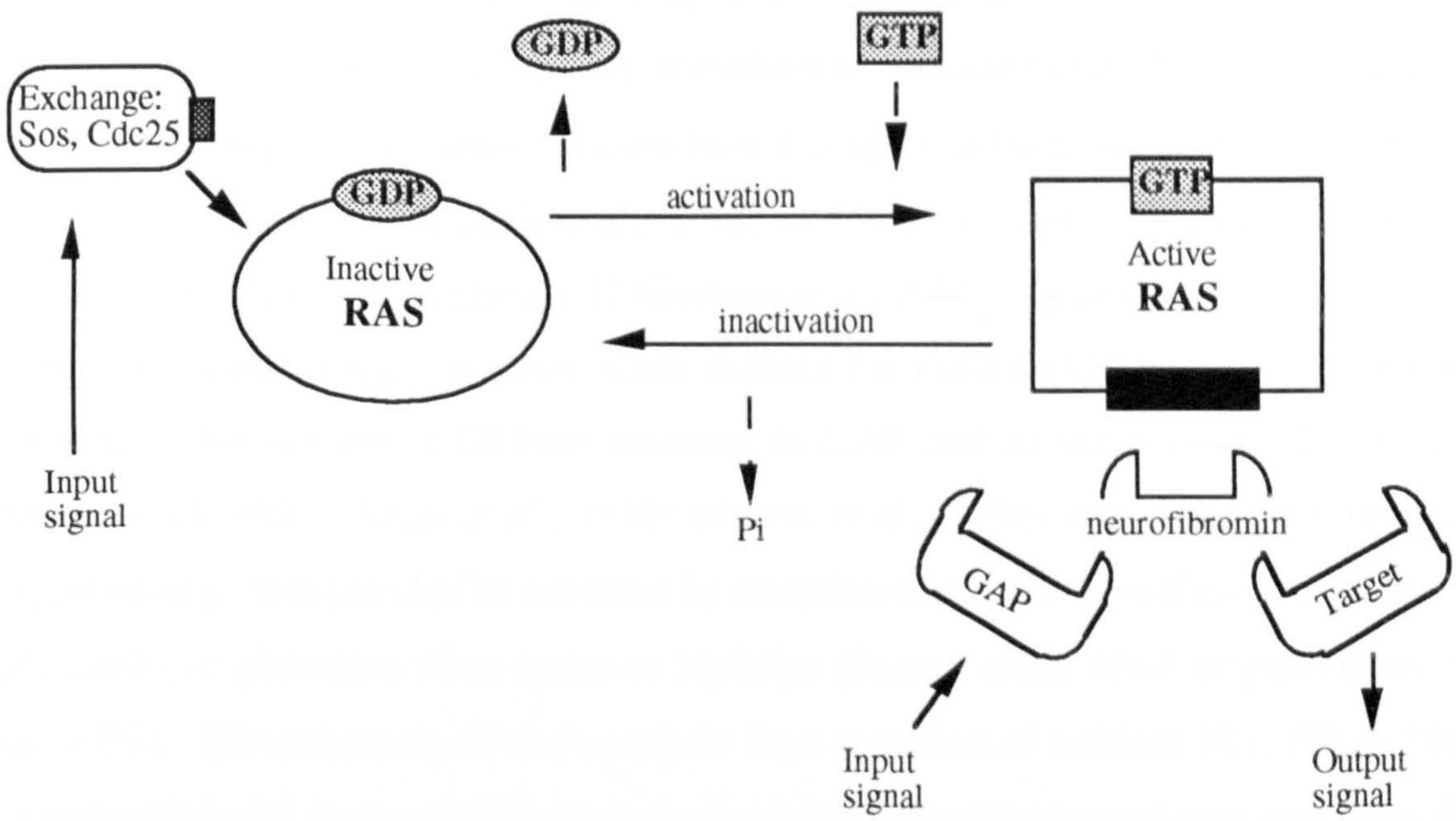


Figure 1.14 Model of ras signalling. Mitogenic signals activate guanine exchange factors and/or GAP, leading to binding of GTP to ras proteins. The GTP-bound activated ras, interacts with target proteins, whose activation results in the cellular changes that end with cell division/differentiation.

The p21 that does dissociate will very rapidly rebind GTP which means that in the normal ras cycle, dissociation of bound GDP leads to the activation of the protein.

Mutations of residues important for contacting the guanine nucleotide can affect the intrinsic dissociation rate, some so much that the rate of spontaneous loading with GTP exceeds the rate of intrinsic GTPase (Noda *et al.*, 1985; Kataoka *et al.*, 1985). Mutations with severe effects on exchange turn out to be activating mutations (Feig & Cooper, 1988b; Sigal *et al.*, 1986; Walter *et al.*, 1986) associated with abnormally high proportion of GTP p21 (Patel *et al.*, 1992; Zhang *et al.*, 1992).

1.7.2.1.3 *Activating mutations*

Mutationally activated (transforming) p21 proteins are associated with alterations in amino acids important for guanine nucleotide co-ordination. Depending on the location of the mutation, the abnormally high proportion of bound GTP results from a decrease in GTPase activity and/or an increase in nucleotide exchange rate. The *ras* oncogenes most commonly isolated from tumours have a single mutation in codon 12, 13 or 61 (Taparowsky *et al.*, 1983; Tabin *et al.*, 1982; Reddy *et al.*, 1982; Yuasa *et al.*, 1983). Substitution of the normal glycine 12 (Seeburg *et al.*, 1984) or glutamine 61 (Der *et al.*, 1986b) by a wide range of amino acids reduces the intrinsic GTPase activity of the protein. This renders it GTPase resistant to GAP and neurofibromin (Trahey & McCormick, 1987; Adari *et al.*, 1988; Martin *et al.*, 1990) and makes the protein transforming. Ras can also be activated by mutation of alanine 59 to threonine (Velu *et al.*, 1989), or glutamine 63 to lysine or histidine (Fasano *et al.*, 1984; Higinbotham *et al.*, 1992). The activation of ras that results from mutation of residues 116, 199 or 146 is associated with a substantial increase in the intrinsic nucleotide exchange rate (Feig & Cooper, 1988b; Sigal *et al.*, 1986). In addition to carrying a codon 12 mutation, both v-H-*ras* and v-K-*ras* encode threonine 59, which (even in the presence of a normal glycine 12) reduces GTPase, reduces sensitivity to GAP, increases exchange and activates transformation (Adari *et al.*, 1988; Gibbs *et al.*, 1984b).

1.7.2.2 *Pancreatic cancer and ras*

K-*ras* mutations occur in pancreatic cancer almost exclusively on codon 12 (Almoguera *et al.*, 1988). These mutations lead to a constitutively active ras protein that is GTPase resistant to GAP and neurofibromin. The most common observed activating mutations in pancreatic cancer are from the wild type glycine (codon 12) to either aspartate, valine or cysteine.

Studies of ductal pancreatic tumours with different degrees of anaplasia have reported that K-ras mutations occur early in pancreatic carcinogenesis (Pellegata *et al.*, 1994). No correlation has been found in adenocarcinoma of the pancreas between the K-*ras* codon 12 genotype and either tumour size or tumour stage (Grunewald *et al.*, 1989; Motojima *et al.*, 1991).

1.7.3 Cytoplasmic & nuclear oncogene products

The scientific research in this area of pancreatic cancer is limited. Experimental evidence suggests that several genes in this group (raf, myc, jun *etc.*) may have important roles in the development of pancreatic cancer but more work needs to be done in this area of pancreatic carcinoma.

1.7.4 Pancreatic cancer and tumour suppressor genes

1.7.4.1 p53

The p53 tumour-suppressor gene encodes a 53 kDa nuclear phosphoprotein which is thought to protect cells against the accumulation of genetic alterations. p53 tumour suppressor gene product plays a critical role in cell cycle regulation and also functions as a nuclear transcription factor. Overexpression of the wild-type p53 protein and an increase in transcriptional activity, following treatment with DNA damaging agents, lead to cell cycle arrest in the G1 phase or the induction of apoptosis (Vogelstein & Kinzler, 1992; Lane, 1993; Levine *et al.*, 1994). Abnormalities in the p53 gene are the most common genetic alteration in human cancer (Hollstein *et al.*, 1991). In normal tissue wild-type p53 protein is difficult to detect, whereas in cells with p53 mutations, conformational changes and a prolonged biological half-life leads to an accumulation of

mutant p53 protein.

In human pancreatic cancer, a high level of p53 expression has been demonstrated in up to 60% of the tumours (Barton *et al.*, 1991b; Scarpa *et al.*, 1993). However, at the present time, results from studies on a possible correlation between overexpression of p53 and survival are inconclusive (Zhang *et al.*, 1994; DiGiuseppe *et al.*, 1994).

1.7.4.2 Deleted in colon cancer (DCC)

DCC as its name implies, is important in the development of colon cancer (Fearon *et al.*, 1990). The DCC gene encodes a protein with multiple immunoglobulin domains and fibronectin type III repeats. In pancreatic cancers the second copy of the DCC gene is frequently mutated (Hohne *et al.*, 1992; Simon *et al.*, 1994). Furthermore, in some pancreatic cancers and their cell lines there is absence or a reduction in DCC expression (Hohne *et al.*, 1992). It has been hypothesised that the loss of this gene may result in pancreatic carcinoma cells that have a more invasive and undifferentiated phenotype.

1.7.4.3 p16/MTS-1 and p15

p16/MTS-1 is a tumour-suppressor gene that is located on chromosome 9p21. The product of this gene is involved in cell cycle regulation. This protein binds specifically to cyclin D-cdk-4 complexes and inhibits cdk-4 mediated phosphorylation of several growth regulatory proteins. Among the species regulated in this manner are RB-1 and the RB-1 related proteins p107 and p130 (Serrano *et al.*, 1993). Pancreatic tumours have been shown to possess mutations in the p16/MTS1 gene (Caldas *et al.*, 1994; Bartsch *et al.*, 1995).There is a higher frequency of mutations in the p16/MTS-1 gene in human pancreatic tumour-derived cell lines compared to primary pancreatic cancers (Huang *et al.*, 1996) as has been found in other tumours.

A closely related gene, p15, was simultaneously identified 25 kb from the p16 gene. Like p16, p15 is involved in the regulation of the cell cycle, by binding to and inhibiting CDK4 and CDK6. The alterations of p16 are more frequent than p15 in cancers. Aberrations have been detected in pancreatic cancer cell lines in the p15 tumour suppressor gene (Naumann *et al.*, 1996).

1.7.4.4 Deleted in pancreatic cancer, locus 4 (DPC4)

A recently discovered tumour suppressor gene, DPC4, is lost or inactivated in 50% of pancreatic cancers (Hahn *et al.*, 1996). However, the function of the DPC4 protein has not been fully elucidated but it resembles the fruit fly protein Mad (mothers against dpp protein) implicated in a transforming growth factor beta-like signalling pathway (Hursh *et al.*, 1993, see Table 1.1 for summary).

The loss of inhibitory influences (p53, p16, p15 and DCC) and the upregulation of stimulatory mechanisms of cell proliferation (growth factors, growth factor receptors and oncogenes) are important mechanisms in the pathogenesis of cancer of the pancreas. In addition to this, other molecular, cellular and environmental changes may contribute to tumour pathogenesis such as gastrointestinal hormones and their receptors and immunological alterations. The cocktail of these changes may give pancreatic cancer cells a major growth advantage and this could lead to the rapid and aggressive spread of the cancer and to unresponsiveness to therapeutic modalities such as chemotherapy and radiotherapy.

Strategies employing gene therapy for pancreatic cancer are being pursued (Lemoine, 1994). The introduction of the p53 gene into colon cancer cells using retroviral vectors resulted in suppressed tumorigenicity of these cells *in vitro* and *in vivo* (Baker *et al.*, 1990). Another approach using antisense oligonucleotides is based on the exogenous administration of short single-stranded oligonucleotide sequences which are thought to act predominantly by blocking translation of mRNA (Carter & Lemoine, 1993; Nellen & Lichtenstein, 1993).

In conclusion growth factor antagonists, gene therapy and antisense oligonucleotides are an attractive and important therapeutic target for pancreatic cancer treatment.

Factor	Status	Reference(s)
Receptors		
MET	overexpression	Di Renzo *et al.*, 1995,
FGFR	overexpression	Kobrin *et al.*, 1993; Ohta *et al.*, 1995
EGFR	overexpression	Korc *et al.*, 1986; Lemoine *et al.*, 1992a
c-*erb*B2	overexpression	Yamanaka *et al.*, 1993c
IGF-IR	overexpression	Bergmann *et al.*, 1995
TGF-βII	overexpression	Friess *et al.*, 1993b
Somatostatin	loss	Buscail *et al.*, 1996
Growth factors		
HGF	overexpression	Ebert *et al.*, 1994; Oikawa *et al.*, 1995
aFGF/bFGF	overexpression	Yamanaka *et al.*, 1993a
EGF/TGF-α	overexpression	Barton *et al.*, 1991a
IGF-I	overexpression	Bergmann *et al.*, 1995
TGF-β1/2/3	overexpression	Friess *et al.*, 1993a
pS2	novel expression	Welter *et al.*, 1992; Collier *et al.*, 1995
Annexin	overexpression	Vishwanatha *et al.*, 1993
Oncogenes		
K-ras	activation	Almoguera *et al.*, 1988; Smit *et al.*, 1988
Tumour suppressor genes		
p53	inactivation	Barton *et al.*, 1991b; Scarpa *et al.*, 1993
DCC	inactivation	Hohne *et al.*, 1992; Simon *et al.*, 1994
p16/MTS-1	inactivation	Caldas *et al.*, 1994; Bartsch *et al.*, 1995
p15	inactivation	Naumann *et al.*, 1996
DPC4	inactivation	Hahn *et al.*, 1996

Table 1.1 The status of various growth factors, growth factor receptors and oncogenes in pancreatic cancer.

1.8 PANCREATIC CANCER AND CHOLECYSTOKININ

In the past few years considerable attention has been given to the possible hormone responsiveness of pancreatic cancer. Much of the work has focused on the gastrointestinal hormone cholecystokinin.

1.8.1 Trophic effects of CCK in the normal gastrointestinal tract

An increase in the size and weight of the pancreas in response to CCK was first described by Rothman & Wells (1967). Other early evidence for hypertrophic and hyperplastic effects of CCK-33, CCK-8 and caerulein on the rat pancreas has been reviewed by Folsch (1984). Similar hypertrophic effects of CCK-8 and caerulein have been described on the pancreas of the Syrian hamsters (Pfeiffer *et al.*, 1982) and mice (Niederau *et al.*, 1987). The effects of exogenous CCK are mediated by the CCK-A receptor, since they can be mimicked by CCK-A receptor agonists and blocked by CCK-A receptor antagonists (Dawra *et al.*, 1993; Povoski *et al.*, 1993a).

The evidence for the role of CCK-A receptor involvement in endogenous CCK in pancreatic growth is not as clear, because the findings vary according to different research workers. Administration of the CCK-A receptor antagonist, L-364,718 to rats by gavage (Wisner *et al.*, 1988) or infusion (Axelson *et al.*, 1990; Gasslander *et al.*, 1990) for 7 weeks caused decreases in pancreatic weight of 21 and 30%, respectively. However, several other groups observed no significant decrease in pancreatic weight during treatment with L-364,718 in rats and similar results have also been reported in guinea pigs and hamsters (Zucker *et al.*, 1989).

Trophic effects of CCK elsewhere in the GI tract are also uncertain. Although there have been reports that CCK-8 and caerulein stimulate epithelial cell proliferation in the mouse gallbladder (Lamote *et al.*, 1982) and that caerulein promotes duodenal hyperplasia in rats (Morisset & Gernik, 1983), continuous subcutaneous infusion of CCK-8 in rats caused no changes in regions of the GI tract other than the pancreas (Axelson *et al.*, 1990).

1.8.2 Experimental proliferative responses of CCK

CCK is involved in the proliferative responses observed in the GI tract following dietary and surgical manipulations of experimental animals as described below. However, gastrin does not appear to be involved.

1.8.2.1 *Protease inhibitors*

The feeding of animals with protease inhibitors leads to pancreatic hypertrophy and hyperplasia (Chernick *et al.*, 1948; Yonezawa, 1983). There is now abundant evidence that this is due to CCK. Circulating levels of CCK are elevated after treatment with protease inhibitors because there is no feedback inhibition of CCK release by excess active trypsin (Niederau *et al.*, 1987). Furthermore, the simultaneous administration of the CCK-A receptor antagonist CR 1409 (Niederau *et al.*, 1987) or L-364,718 (Wisner *et al.*, 1988) markedly reduces the hypertrophic response .

1.8.2.2 *Surgical treatments*

CCK is also a factor responsible for pancreatic hypertrophy and hyperplasia following pancreaticobiliary diversion (PBD) in both freely fed and fasting rats (Gasslander *et al.*, 1990; Chen *et al.*, 1993a). PBD in the rat and hamster is achieved by transferring the part of the duodenum containing the pancreatic and bile ducts to the middle of the ileum. The operation results in elevated serum levels of CCK (Gasslander *et al.*, 1990) and decreased serum levels of gastrin (Miazza *et al.*, 1985; Chu *et al.*, 1992). The increase in pancreatic weight by PDB is blocked by CCK-A receptor antagonists (Axelson *et al.*, 1990; Gasslander *et al.*, 1990; Rivard *et al.*, 1991). A similar association between plasma CCK levels and increased pancreatic growth has been observed following cholecystectomy in Syrian hamsters (Rosenberg *et al.*, 1988).

1.8.2.3 *Pancreatitis*

CCK has also been implicated in pancreatic regeneration following pancreatitis induced by caerulein or ethionine. Treatment with exogenous CCK-8 accelerated regeneration following ethionine-induced pancreatitis, while spontaneous regeneration following caerulein-induced pancreatitis was inhibited by L-364,718 (Jurkowska *et al.*, 1992).

1.8.3 Effects of CCK on spontaneous carcinogenesis

At least one surgical treatment known to increase serum CCK levels may also enhance spontaneous pancreatic carcinogenesis. PDB resulted in the appearance of hyperplastic nodules in the pancreas of over 70% of treated rats (Miazza *et al.*, 1987; Stace *et al.*, 1987). Similar results were obtained when serum CCK was elevated by feeding rats on raw soya flour (Roebuck *et al.*, 1987; Morgan *et al.*, 1977; McGuiness *et al.*, 1980), but no hyperplastic nodules were observed in mice fed on the protease inhibitor, camostate (Niederau *et al.*, 1990).

1.8.4 Effects of CCK on chemically-induced carcinogenesis

Exogenous CCK and caerulein enhance chemically-induced carcinogenesis in the pancreas (Lamers *et al.*, 1988). Both CCK-8 and caerulein stimulated the azaserine initiated development of acidophilic atypical acinar cell nodules (AACN), which are preneoplastic lesions in the rat pancreas (Douglas *et al.*, 1989; Lhoste & Longnecker, 1987). It has been demonstrated that azaserine-induced AACN contain a 6 fold higher concentration of high affinity CCK receptors than internodular tissue (Bell *et al.*, 1992), and these receptors mediate the trophic actions of sCCK-8 since the effects were duplicated by a selective CCK-AR agonist (A-71623), but not by a selective gastrin/CCK-B receptor agonist (SNF-8815, Povoski *et al.*, 1993a). In contrast to the enhancement of acinar tumour development by CCK, pancreatic ductular adenocarcinoma induced by *N*-nitroso (2-oxopropyl) amine (BOP) in hamsters is unaffected by treatment with CCK or caerulein following carcinogen treatment (Andren-Sandberg *et al.*, 1984; Pour *et al.*, 1988).

Elevation of CCK by surgical manipulation also enhances chemically-induced pancreatic carcinogenesis. PDB or partial gastrectomy both increased serum CCK in rats and the number and volume of acidophilic AACN were increased (Watanapa *et al.*, 1992; 1993; Stewart *et al.*, 1991). Furthermore, the CCK-AR is presumably involved, since the number of AACN was reduced by 90% following treatment with the CCK-AR selective antagonist (CR 1409) (Watanapa *et al.*, 1993). In another model, cholecystectomy also increased serum CCK levels and doubled the incidence of BOP-induced pancreatic ductular carcinomas in hamsters (Ura *et al.*, 1986).

Chemically-induced pancreatic carcinogenesis is also promoted by feeding protease inhibitors to rats. Increased numbers of AACN were observed in rats fed raw soya flour and treated with either azaserine (Roebuck *et al.*, 1987; Morgan *et al.*, 1977; McGuiness *et al.*, 1981) or BOP (Levison *et al.*, 1979). The same increase was seen after feeding camostate to azaserine-treated rats (Lhoste *et al.*, 1988). This effect was significantly reduced by treatment with the CCK-A receptor antagonist CR 1409.

1.8.5 *In vivo* tumour xenograft models

1.8.5.1 *Immunodeficient mice*

Researchers have attempted to transplant tumours from one species to another for more than a century, the majority of such attempts was aimed at transferring human tumours into other species to allow controlled experimental investigations. However, this proved to be very difficult until genetically immunosuppressed animals were available. Since then, immunodeficient animals have played an important role in the studies of tumours *in vivo*.

1.8.5.1.1 *Nude mice*

In 1966, a spontaneous hairless mouse was born in an animal colony in Glasgow, Scotland (Flanagan, 1966) and subsequently this mutant strain was successfully bred. It was noted that the mutant mouse had a greatly reduced fertility, failed to thrive and developed severe infections. These mice lack a normal thymus. The name 'nude' was adopted for this mutation which is inherited in an autosomal recessive pattern. Nude mice have normal precursors of B and T cells. However, the abnormal development of the thymic epithelium is incapable of inducing normal differentiation of T cells. Therefore, nude mice are severely depleted of thymic-derived lymphocytes. Characteristics of nude mice include failure to reject xenografts, lack of helper and suppressor T cell activity, inability to mount graft versus host response and inability to generate cytotoxic T cells.

1.8.5.1.2 *SCID mice*

In 1981, a spontaneous mutation arose in a breeding colony of C.B-17 mice at Fox Chase Cancer Center and was first described by Bosma *et al.* (1983). The mutant allele SCID (severe combined immunodeficient) is inherited in an autosomal recessive pattern. This mouse is more severely immunocompromised than the nude mouse, lacking both functional B and T cells because of non-functional rearrangements of immunoglobulin and T-cell receptor genes (Schuler *et al.*, 1986). However, natural killer cells, macrophages and other haemopoietic cell lineages do not appear to be affected by the autosomal recessive SCID mutation (Dorshkind *et al.*, 1985). Although the SCID mutation affects only lymphocyte development, it is not clear what stage of differentiation is impaired. It is thought that the effects of the SCID mutation become manifest after commitment of lymphoid cells to the B and T cell pathways.

These mice are now used widely to study tumour biology, biochemistry and experimental therapy. Tumours can be transplanted from primary tumour material or from tumour cell lines (Rygaard & Povlsen, 1969, Giovanella *et al.*, 1972).

1.8.6 Effects of CCK on tumour growth *in vivo*

There are a number of reports on the effects of CCK and its analogues on xenograft growth. In some studies exogenous CCK and caerulein have been shown to increase tumour weight and DNA content in xenografts of pancreatic tumour cell lines (Smith *et al.*, 1990a; Roebuck *et al.*, 1987; Upp *et al.*, 1987). Caerulein injections induced the growth of pancreatic tumour (SKI with CCK-R) xenografts in nude mice (Upp *et al.*, 1987) and the CCK-9 analogue increased the growth of SW1990 cell line xenografts in nude mice (Smith *et al.*, 1990a, 1991). No effects of exogenous cholecystokinin were seen with the human pancreatic cancer cell line xenografts of CaV (Upp *et al.*, 1987), Mia PaCa-2, Panc-1, P1420 (Hudd *et al.*, 1989, 1985), PGER (Maani *et al.*, 1988), or with xenografts of PC-T1 (Nio *et al.*, 1993). A decrease in the volume of PC-YY xenografts following CCK injections has also been observed (Nio *et al.*, 1993). The combination of caerulein and secretin also increased tumour weight and DNA content of the pancreatic ductular adenocarcinoma H-2-T in hamsters (Townsend *et al.*, 1981) and SW1990 in mice (Smith et *al.*, 1990a).

CCK-R antagonists have been used to investigate the role of pancreatic cancer *in vivo*. These antagonists have been given in combination with exogenous CCK to demonstrate that changes associated with CCK administration are specific receptor-mediated effects. Antagonists have been used alone to investigate the role of endogenous CCK in pancreatic cancer. Proglumide was shown to inhibit the increases in caerulein-induced tumour size, weight, protein and RNA in xenografts of the human SKI cell line (Upp *et al.*, 1987). Loxiglumide caused the inhibition of the tumour growth ratio of PC-T1 cell xenografts in nude mice (Nio *et al.*, 1993). L-364,718 reversed the growth effects of CCK-9 in SW1990 cell xenografts (Smith *et al.*, 1990b). Aspercilin and devazepide have been shown to reduce tumour size of xenografted tumours in nude mice (Alexander *et al.*, 1987; Maani *et al.*, 1988).

Some experiments that are difficult to explain have shown stimulatory effects of both cholecystokinin and cholecystokinin antagonists on pancreatic cancer when used alone, but no stimulation when these are used in combination (Smith *et al.*, 1990b).

1.8.7 Effects of CCK on tumour growth *in vitro*

In vitro cell culture studies on the trophic effects of exogenous CCK have given rise to conflicting results. CCK has been shown to stimulate growth of the human pancreatic cancer cell line, KP-1N (Funakoshi & Kono, 1992), and to increase the uptake of [^{3}H]-thymidine in the PC-F3 cell line, derived from ascites in a patient with pancreatic adenocarcinoma (Morimoto *et al.*, 1993). The growth of six human pancreatic cancer cell lines (SW-1990, Panc-1, Mia PaCa-2, BxPc-3, RWP-2 and Capan-2) was stimulated by CCK (Smith *et al.*, 1990a; Smith *et al.*, 1991; Smith *et al.*, 1993) in either the sulphated or unsulphated form (Heald *et al.*, 1992). CCK also stimulated the growth of the rat pancreatic carcinoma cell line, AR42J (Scemama *et al.*, 1989). However, other experiments have shown no effect of these peptides on tumour cell growth *in vitro* and some studies have even shown inhibition (Morimoto *et al.*, 1993; Liehr *et al.*, 1990; Nio *et al.*, 1993). Liehr *et al.* (1990) observed no change in DNA, protein or cloning efficiency in Panc-1 and Mia PaCa-2 cells after incubation with CCK-8 or a CCK-9 analog.

Conflicting results have been published from *in vitro* experiments designed to determine the effects of various CCK-R antagonists on the growth of pancreatic cancer cells in the absence of exogenous CCK. Some studies have shown no effect of CCK-R antagonists on growth. In an experiment using serum-free media without exogenous CCK, Smith *et al.* (1993) found that devazepide and L-365,260 had no effect on the growth of the human Panc-1 cell line. Studies by Morimoto *et al.* (1993) with the human pancreatic cancer cell line PC-HN *in vitro* also showed no effect of devazepide on growth. Earlier studies have shown stimulation of pancreatic cancer cells by receptor antagonists (Smith *et al.*, 1990b) but other *in vitro* experiments show inhibition of pancreatic cancer cell lines by CCK-R antagonists in the absence of exogenous CCK. Loxiglumide inhibited [^{3}H]-thymidine uptake in serum-free medium without exogenous CCK in various human pancreatic cancer cell lines (Morimoto *et al.*, 1993; Nio *et al.*, 1993).

1.9 PANCREATIC CANCER AND GASTRIN

1.9.1 Trophic effects of gastrin in the normal gastrointestinal tract

The observation that the removal of the source of gastrin by antrectomy led to atrophy of the remainder of the gastric mucosa was the first indication that gastrin was involved in the control of gastric mucosal growth (Lees & Grandjean, 1968; Gjurldsen *et al.*, 1968). In the rat this atrophy could be reversed by the administration of pentagastrin (Johnson & Chandler, 1973). Conversely, the elevated levels of serum gastrin which are a characteristic of patients with the Zollinger-Ellison syndrome, lead to gastric mucosal hyperplasia (Neuberger *et al.*, 1972) which can be reversed by the surgical removal of the gastrinoma (Pisegna *et al.*, 1992b). There is now general agreement that in fed animals gastrin stimulates the growth of the gastric mucosa only (Ryberg *et al.*, 1990; Crean *et al.*, 1969).

1.9.2 Experimental proliferative responses of gastrin

1.9.2.1 *Small bowel resection*

The adaptation of the residual small bowel following the resection of the small intestine was not prevented by the removal of the source of gastrin by antrectomy (Oscarson *et al.*, 1977) or gastrectomy (Tilson & Axtmayer, 1976). Pancreatic hyperplasia observed in rats after small intestine resection is not a consequence of hypergastrinaemia as it was unaltered by prior antrectomy or vagotomy.

1.9.3 Effects of gastrin on spontaneous carcinogenesis

Although gastrin does not appear to have any effects on the rate of spontaneous pancreatic carcinoma development, there is now evidence to show that elevated serum gastrin increases the frequency of gastric carcinoid tumours in rats (Tielemans *et al.*, 1990; Brenna & Waldum, 1992).

1.9.4 Effects of gastrin on chemically-induced carcinogenesis

The effects of gastrin in chemically-induced pancreatic carcinogenesis have not been determined. However, the effects of gastrin on gastric and colonic cancer induced by mutagens is still controversial. When given together with the mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), pentagastrin increased the incidence of gastric carcinoma in rats (Tahara & Haizuka, 1975) and dogs (Kurihara *et al.*, 1979). However, when G-4 was administered after the mutagen, the incidence of gastric carcinoma was reduced (Tatsuta *et al.*, 1980). Similar confusions exist on the effect of gastrin on colonic carcinogenesis. Subcutaneous injection of G-4 has been reported to reduce the incidence of MNNG-induced colonic carcinomas in rats (Tatsuta *et al.*, 1983), conversely reduction of serum gastrin levels by feeding rats a chemically defined diet resulted in an increased incidence of azoxymethane-induced colonic carcinoma (Tatsuta *et al.*, 1985).

1.9.5 Effects of gastrin on tumour growth *in vivo*

Compared to CCK very little work has been done on the effects of gastrin on the growth of pancreatic cancer both *in vivo* and *in vitro*. However, recently it has been shown that pentagastrin stimulated the growth of pancreatic tumour cell line xenografts in nude mice. This was shown by an increase in volume, DNA and protein content of the xenografted tumours and the growth response was inhibited by a CCK-BR antagonist, L-365,260 (Smith *et al.*, 1995).

1.9.6 Effects of gastrin on tumour growth *in vitro*

Gastrin promotes the growth of pancreatic tumour cell lines in culture (Smith *et al.*, 1995) and the rat pancreatic tumour cell line AR42J has also been shown to respond to gastrin by an increase in DNA synthesis (Seva *et al.*, 1990b).

1.10 AIMS

The aim of these studies is to determine the role of CCK-A and CCK-B receptors in the growth of human pancreatic cancer combining three different areas of work; a) molecular determination of the expression of CCK receptors in human pancreatic cancers and cell lines b) *in vitro* cell culture studies to evaluate CCK agonist and antagonists effects on tumour growth c) *in vivo* xenograft studies to evaluate the effects of CCK agonist and antagonists on human pancreatic tumour growth.

MATERIALS AND METHODS

CHAPTER 2

CELL CULTURE STUDIES

CHAPTER 2

2.1 Cell culture

Human pancreatic tumour cell lines were grown in 175 cm^2 tissue culture flasks in a sterile incubator (Leec) at 37°C with 10% carbon dioxide under the following growth conditions:-

Mia-PaCa-2 : 10% foetal calf serum (FCS) and 2.5% horse serum in Dulbecco's modified eagle's medium (DMEM) with sodium bicarbonate.

BxPc-3, Capan-2 , Panc-1 : 10% FCS in RPMI 1640.

Capan-1 : 15% FCS in RPMI 1640.

AsPc1 : 20% FCS in RPMI 1640 containing 100 U/ml penicillin and streptomycin.

Hs766T : 10% FCS in DMEM with sodium bicarbonate.

KPan : 10% FCS in DMEM/Ham's F12 with sodium bicarbonate supplemented with 15 mM HEPES, l6 nM epidermal growth factor, 1.4 µM hydrocortisone, 1 µM insulin, 5.0 µM isobutylmethylxanthine and 5 mM glutamine.

Jurkatts (J6, T-lymphoma cell line) : 10% FCS in RPMI 1640.

NIH3T3: 10% bovine calf serum in DMEM

NIH3T3CCK-BR: 10% bovine calf serum in DMEM.

All culture medium contained 100 U/ml of penicillin and streptomycin.

When the cells reached 70% confluency (between 5-12 days) tissue culture medium was decanted and 4 ml of trypsin added to the cells for 5 min at room temperature. Once the cells were detached from the flask the appropriate medium (as above) for each cell line was mixed into the trypsinised cells. A sample of the cells were counted using trypan blue (Sigma).

All materials, kits and solutions for these cell culture experiments are contained in appendix I, p 277-279.

2.2 Cell counting

50 µl of the trypsinised cells were added to an equal volume of trypan blue, mixed and applied to a haemocytometer slide. Using the the trypan blue exclusion technique dead cells were stained blue but the unstained live cells were counted as follows:

Total number of live cells = Cells counted x dilution factor (2) x $1x10^4$ x total volume

The cells were split into a number of flasks ($4 x10^4$ cells per flask) topped up with growth medium and incubated at 37°C in 10% carbon dioxide under sterile conditions. This procedure was repeated as required.

2.3 Freezing and reconstitution of cells

Cells grown in culture were sometimes stored frozen in a freezing medium containing 10% DMSO (Sigma) in foetal calf serum. The cells were harvested by trypsinisation and centrifugation then resuspended in the freezing medium at a density of $1x10^6$ cells/ml, in 1 ml aliquots in cryovials. The vials were placed in a cell freezing unit, Mr. Frosty ™ (Sigma), containing isopropanol and cooled in a -70°C freezer overnight and subsequently stored in a liquid nitrogen safe.

Frozen stocks were reconstituted by thawing a cryovial at 37°C and the cells were then gently transferred into Falcon tubes (14 ml) containing 10 ml nutrient medium at room temperature. The cells were centrifuged for 10 min at room temperature at 130 x *g* (super minor centrifuge, Fisons). The cells were again resuspended in nutrient medium at $4 x 10^3$ cells per flask and given fresh nutrient medium every 2 days.

2.4 Growth of Mia PaCa-2 and BxPc-3 cell lines

The cells were plated out in 24 well plates at a cell density of $0.3 x 10^4$ cells per well in 1.0 ml of the appropriate tissue culture medium as described in 2.1. 1 plate of cells from each cell line was harvested for cell counting every 24 hr by aspirating the spent medium and adding 100 µl of trypsin per well for 5 min. The cells were counted using

the trypan blue technique 2.2.

2.5 The effects of sCCK-8 and nsG-17 on the growth of Mia PaCa-2 and BxPc-3 cell lines

The cells were plated out as described in section 2.4 in FCS for 5 days (*i.e.* allowed to reach log phase of growth). Following this incubation the cells were washed twice with serum free medium and incubated for a further 4 days in serum-free medium before stimulating with growth agents. The cells were stimulated with various concentrations of i) sCCK-8 ii) nsG-17 (10 pM, 0.1 nM, 1 nM, 10 nM and 100 nM) iii) 10% foetal calf serum for varying lengths of time. The medium plus growth factors were replaced every 24 hr but those cells to be counted were harvested every 48 hr with 100 µl of trypsin per well for 5 min and counted using trypan blue.

2.6 The effects of CCK-AR and CCK-BR antagonists on the growth of Mia PaCa-2 and BxPc-3 cell lines

The cells were plated out as described in section 2.4 in FCS and then in serum-free medium. Following the 4 day incubation in serum-free medium the cells were incubated in medium containing either i) 10% foetal calf serum ii) 10% foetal calf serum in the presence of varying concentrations of antagonists (1 nM, 100 nM and 10 µM) iii) serum-free medium iv) serum-free medium in the presence of varying concentrations of antagonists (1 nM, 100 nM and 10 µM). The medium was replaced every 24 hr. On day 6 (post-antagonist incubation) the cells were harvested with trypsin and counted utilising trypan blue. The following antagonists were investigated; RPR-X, L-740,093, L-365,260, L-364,718 (devazepide), CR 1409 (lorglumide). These antagonists were made in sterile 100% DMSO at a concentration of 100 µM except the antagonist L-740,093 which was dissolved in phosphate buffered saline (PBS) at 100 µM. The vehicle effect was also determined by incubating the cells with the vehicle in medium.

2.7 The effects of sCCK-8 and nsG-17 on the growth of NIH3T3CCK-BR cells

NIH3T3CCK-BR cells were plated out at 1 x 10^4 cells per well in 24 well plates in DMEM containing either 10% bovine calf serum, sCCK-8 or nsG-17 at various concentrations (10 pM, 100 pM, 1 nM, 10 nM and 100 nM). The medium was replaced every 24 hr. The cells were counted every 48 hr by trypsinisation for 5 min followed by counting (trypan blue technique).

2.8 The effects of CCK-AR and CCK-BR antagonists on the sCCK-8 induced growth response in NIH3T3CCK-BR cells

NIH3T3CCK-BR were plated out at 1 x 10^4 cells per well in 24 well plates in DMEM tissue culture medium containing either 10% bovine calf serum, 100 nM sCCK-8 in the presence or absence of varying concentrations of antagonists (1 pM, 0.1 nM and 10 nM). The medium was replaced every 24 hr and the cells counted on day 8. The antagonists investigated were ; CI988, RPR-X, L-740,093, L-365,260, L-364,718 (devazepide), CR 1409 (lorglumide). The vehicle effect was also determined.

CHAPTER 3

FAK PHOSPHORYLATION STUDIES

CHAPTER 3

3.1 Stimulation of cells

NIH3T3CCK-BR cells were plated out at $1x10^6$ per petri-dish in 10 ml DMEM containing 10% calf serum (bovine donor) and 100 U/ml penicillin and streptomycin. The cells were grown for 48 hr before replacing the medium with serum free DMEM containing low sodium bicarbonate. 48 hr later the cells were stimulated for 5 min with 100 nM sCCK-8, 100 nM nsG-17, 10% calf serum and PBS as control in a sterile incubator at 37°C in 10% carbon dioxide.

Mia PaCa-2 cells were plated out at $1x10^6$ per petri-dish in 10 ml DMEM containing 10% FCS, 2.5% horse serum and 100 U/ml penicillin and streptomycin. The cells were grown for 48 hr before replacing the medium with serum free DMEM containing low sodium bicarbonate. 48 hr later the cells were stimulated for 5 min with varying concentrations of sCCK-8 and nsG-17 (10 nM, 50 nM, 200 nM and 500 nM), 10% calf serum and PBS as control in a sterile incubator at 37°C in 10% carbon dioxide.

In order to determine the effects of CCK-R antagonists on basal FAK phosphorylation in Mia PaCa-2 cells, the cells were plated out in FCS containing medium as above in the presence of various antagonist concentrations (0.1 nM, 10 nM and 1 μM). The cells were grown for 48 hr before replacing the medium with serum free DMEM (containing low sodium bicarbonate) containing the appropriate concentration of antagonist for 48 hr.

All materials and solutions for these studies are contained in appendix I, p279-281.

3.2 Preparation of cell extract for protein analysis

Following the 5 min stimulation with the agonists and 48 hr incubation with the antagonists, the medium was rapidly aspirated and the cells were washed once with 5 ml of 2 mM sodium vanadate. The cells were lysed with 500 μl of immunoprecipitation (IP) buffer and the lysate scraped into a clean eppendorf with the

aid of a cell scraper. The cell lysate was agitated on a rotating wheel for 30 min at 4°C then microfuged at 6500 x *g* for 15 min. The resulting supernatant was transferred to a fresh eppendorf. The concentration of protein in the sample was determined by the Bradford assay.

3.3 Bradford Assay

5.0 ml of Bradford solution was aliquoted into bijoux bottles to which varying amounts of bovine serum albumin was added (0.5 µg-10.0 µg, used as standards). 2.0 µl of sample was added to 5.0 ml of the Bradford solution. The sample plus Bradford solution was allowed to stand at room temperature for 30 min before taking the absorbance readings at a wavelength of 595 nm.

3.4 Preparation of protein A beads and primary antibody

250 mg of protein A beads (Sigma) were resuspended in 9 ml of phosphate buffered saline (PBS containing 0.1% BSA, 0.01% azide, 0.5% NP40 and 0.1% SDS) and agitated at 4°C for 30 min (binding capacity = 20 mg of human IgG/ml of beads). The beads were stored at 4°C and washed with PBS prior to use. 100 µl of protein A beads were used per sample to be immunoprecipitated. 10 µg of rabbit anti-mouse IgG (Zymed) was added to 100 µl of beads and incubated overnight at 4°C. The beads were then centrifuged at 6500 x *g* for 30 sec, the supernatant discarded and the pellet resuspended in 500 µl of IP buffer, mixed and centrifuged again. In total, the beads were washed 3 times and finally resuspended in 100 µl of IP buffer. 2.5 µg of FAK antibody (Affiniti) was added to the beads and mixed at 4°C for 30 min. Following this incubation, the beads were washed 3 times with IP buffer as before. After the third wash the beads were resuspended in 500 µl of 2x IP buffer.

3.5 Immunoprecipitation of FAK

250 µg of cell lysate protein (approximately 250 µl) was added to the above prepared protein A beads (bound to IgG-FAK antibody). The final volume of the immunoprecipitation step was made up to 1 ml with sterile distilled water (250 µl). This cocktail was agitated for 1.0 hr at 4°C. The immunoprecipitate was centrifuged at 6500 x *g* for 4 min and the supernatant discarded. The pellet was washed twice with

1x IP buffer. Following the second wash the beads were resuspended in 50 µl of 2x sample buffer. The samples were immediately subjected to electrophoresis.

3.6 Preparation of SDS-polyacrylamide gels for protein separation

For each gel, a pair of front and back plates were washed with Hibiscrub and water. The plates were rinsed with distilled water and dried with a lint-free paper towel and finally wiped with 70% ethanol. The gel apparatus was assembled according to the manufacturer's instructions (Anachem, 17.5 x 20.5 cm) with 0.4 mm uniform thickness spacers and clamped. 6.5% polyacrylamide solution mix was made to which the TEMED was added just before pouring the gel between the plates. The separating gel was introduced in between the plates using a syringe, leaving sufficient space above for the stacking gel. The separating gel was overlaid with 70% ethanol and allowed to polymerise for 30 min. The overlaying solution was drained by inverting the apparatus and the gel rinsed with ddH_2O to remove any unpolymerised acrylamide. The remainder was filled with the 3.5% polyacrylamide stacking gel mix and the comb inserted immediately. The gel was allowed to set for 30 min. The comb was then gently removed, to avoid stretching of the gel and the wells rinsed with 1x running buffer. The bottom reservoir of each gel apparatus was filled with 1x running buffer so that the gels would be submerged 2-3 cm in buffer. Each gel sandwich was unclamped, placed in a Western electrophoresis apparatus and clamped. 1x running buffer was poured into the top reservoir filling the wells.

3.7 Electrophoresis of protein gels

The immunoprecipitated samples with the protein A beads were heated at 100°C for 5 min. The boiled samples were centrifuged at 6500 x *g* for 4 min and 50 µl of the supernatant loaded into each well. The samples were subjected to electrophoresis at 50 mA until the bromophenol blue tracking dye had entered the separating gel. The current was then increased to 55 mA and electrophoresis continued for approximately 3 hr.

3.8 Electroblotting of proteins

Following electrophoresis the gel was dismantled and cut, removing the stack and unrequired regions of the gel. Nitrocellulose membrane (Schleicher and Schuell) was cut according to the size of the gel and immersed in 1x transfer buffer for 5 min with gentle agitation. Whatman 3MM paper (x2) was also cut to the size of the gel and soaked in 1x transfer buffer. The components were set up for transfer by sandwiching the gel and nitrocellulose membrane between 2 pieces of Whatman 3MM filter paper, and then between two Scotch-Brite pads (20 x 20 cm and 0.5 cm thick). These were placed in a plastic transfer cassette and lowered into the electroblotting apparatus (BioRAD) in correct orientation with the membrane on the anode (positively charged) side of the tank. The tank was topped up with 1x transfer buffer and the proteins were electrophoretically transferred from gel to membrane overnight at 200 mA at 4°C. After electroblotting the membrane was used for immunoprobing whereas the gel was Coomassie Blue stained to verify the transfer of proteins onto the membrane.

3.9 Immunoprobing for phosphorylated proteins

The membrane with the blotted proteins was cut so that one lane containing the immunoprecipitated FAK was removed from the rest of the blot enabling the protein in this well to be probed with FAK antibody while the remainder was probed with phosphotyrosine antibody (PY20, Affiniti, U.K).

The membrane was washed in TBS for 10 min with agitation at room temperature and transferred to a plastic container with blocking buffer (2% BSA in 0.1% TBST) and agitated for 1 hr at room temperature on a shaker. The membrane was rinsed with 0.1% TBST and incubated in 0.1% TBST containing the primary antibody, 0.4 µg/µl PY20 for a further 1 hr with agitation at room temperature. The PY20 solution was discarded and the membrane washed 6 times for 5 min in 0.1% TBST with constant agitation at room temperature. The secondary antibody, 0.5 ng/µl horse-radish peroxidase (DAKO) in 5% powdered milk in 0.1% TBST was added to the membrane and incubated for 45 min at room temperature with shaking. Following this, the membrane was washed 6 times for 5 min in 0.1% TBST with constant agitation at room temperature. Finally the membrane was rinsed in double distilled water (ddH_20)

for 10 min.

3.10 Immunoprobing for FAK protein

The membrane was washed in TBS for 10 min with agitation at room temperature and transferred to a plastic container with blocking buffer (5% powdered milk in 0.1% TBST), incubated for 1 hr at room temperature on a shaker. The membrane was rinsed with 0.1% TBST and incubated in 0.1 %TBST containing the primary antibody, 0.25 µg/µl FAK antibody (Affiniti, U.K) for a further 1 hr with agitation at room temperature. The FAK antibody solution was discarded and the membrane washed 6 times for 5 min in 0.1% TBST with constant agitation at room temperature. The secondary antibody, 0.5 ng/µl horse-radish peroxidase in 5% powdered milk in 0.1% TBST was added to the membrane and incubated for 45 min at room temperature with shaking. The membrane was washed 6 times for 5 min in 0.1% TBST with constant agitation at room temperature (see Figure 3.1). Following this the membrane was rinsed in ddH_2O for 10 min. The original PY20 blot was put together with the lane probed with the FAK antibody and the proteins were detected by enhanced chemiluminescence (ECL, Amersham) using an exposure time of 30 sec.

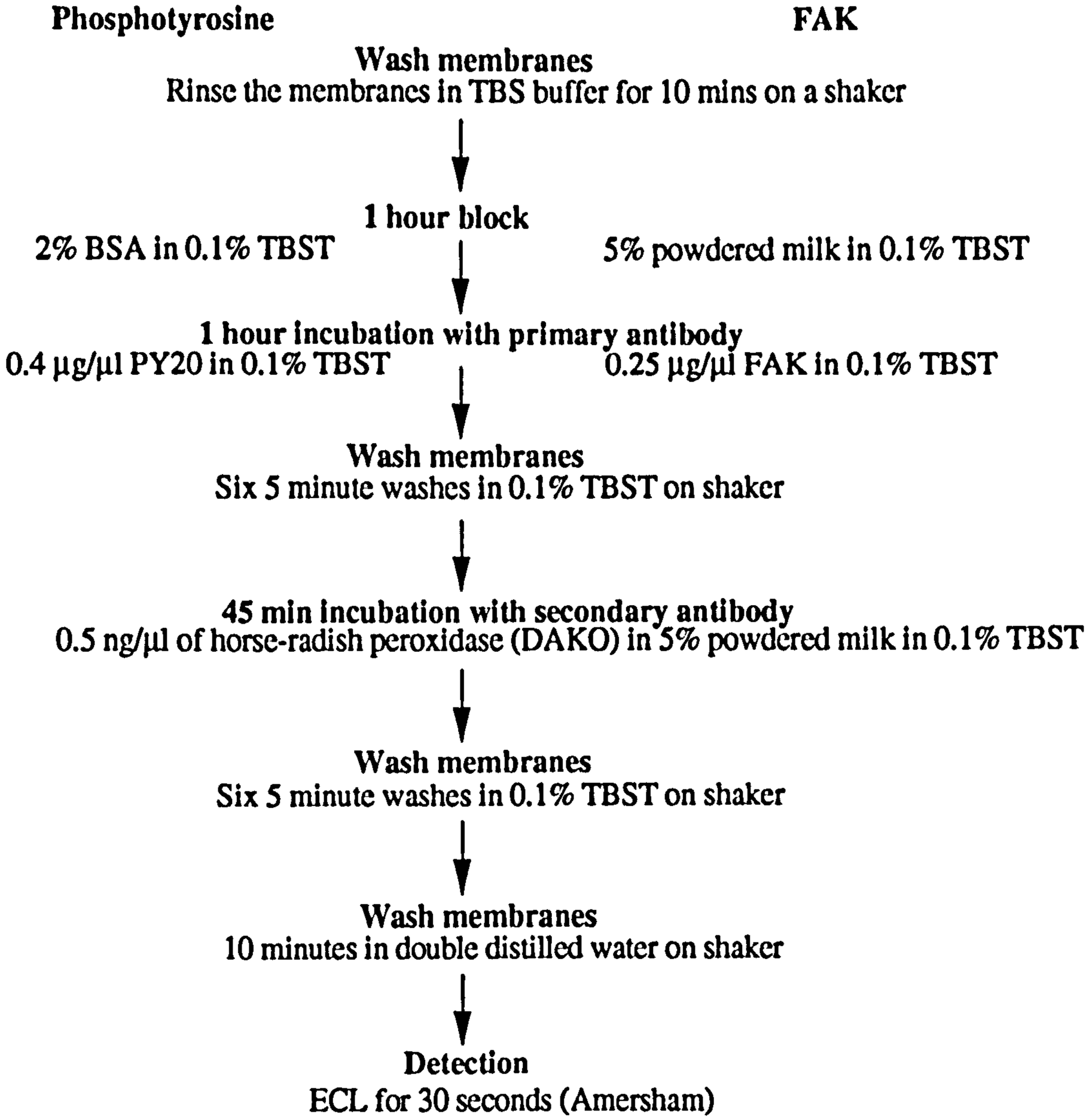


Figure 3.1 Flow diagram summarising the immunoprobing procedures using PY20 and FAK antibodies.

CHAPTER 4

MOLECULAR STUDIES

CHAPTER 4

4.1 Determination of CCK-R expression in human pancreatic cancer cell lines by PCR.

4.1.1 Cell culture

All chemicals, solutions and kits for these molecular studies are referred to in appendix I, p277-287. Eight human pancreatic tumour cell lines (Mia-PaCa-2 , Panc-1, Capan-2 , BxPc-3 , Capan-1 , AsPc-1, Hs766T and KPan) and Jurkatts (J6, T-lymphoma cell line) were grown in the appropriate medium (as described in 2.1) in tissue culture flasks in a sterile incubator at 37°C in the presence of 10% carbon dioxide. When the cells reached 70% confluency, total ribonucleic acid (RNA) was extracted from the cells.

4.1.2 Isolation of total RNA from cultured cells

Total ribonucleic acid (RNA) was extracted from each of the pancreatic tumour cell lines by the method of Chomczynski and Sacchi (1987) using RNAzol (Biogenesis, U.K). At 70% confluency tissue culture medium was discarded and the cells trypsinised with 5 ml of 1x trypsin (Difco) until complete detachment of the cells from the flask was achieved (approximately 5 min). The cells were transferred into 50 ml falcon tubes (Corning) and the trypsin inactivated by the addition of culture medium containing serum. The cells were centrifuged at 130 x *g* for 10 min. The supernatant was aspirated and 3 ml of RNAzol added to the cell pellet (approximately 1 x 10^6 cells) which were lysed by vigorous vortexing. Chloroform (0.3 ml, a tenth of the volume of RNAzol) was added and the mixture vortexed and placed on ice for 5 min. The suspension was centrifuged in a Sorvall RC-5 superspeed refrigerated centrifuge at 12,000 x *g* for 15 min. The top aqueous phase was transferred to a sterile tube and an equal volume of propan-2-ol (BDH) added and RNA precipitated overnight at -20°C. The precipitated RNA was centrifuged at 4°C for 15 min at 12,000 x *g*, the supernatant was decanted and the RNA pellet washed in 70% ethanol and centrifuged again at 6000 x *g* for 10 min at 4°C. The supernatant was decanted and the pellet resuspended in an

appropriate volume (50-150 µl) of diethylpyrocarbonate (DEPC) treated water.

4.1.3 Isolation of total RNA from tissues

All the apparatus used was pre-cooled, the Wheaton homogenisers were stored at 4°C and the metal pounders were supercooled at -70°C for a minimum of 15 min. Frozen tissue (200 mg) was rapidly ground into powder form by hammering the pounder while keeping the tissue frozen with liquid nitrogen. The resultant ground tissue was rapidly transferred to a homogeniser containing RNAzol B (3 ml) and homogenised on ice (5 min). The homogenised tissue was transferred to a clean snap-top tube (Greiner) and 500 µl of chloroform (a tenth of the volume of RNAzol) was added and the mixture vortexed and held on ice for 5 min. The cells were centrifuged in a Sorvall RC-5 superspeed refrigerated centrifuge at 12,000 x *g* for 15 min. The top aqueous phase was transferred to a sterile tube and an equal volume of isopropanol added and RNA precipitated overnight at -20°C. Following the precipitation, the RNA was centrifuged at 4°C for 15 min at 12,000 x *g*, the supernatant was decanted and the RNA pellet washed in 70% ethanol at 6000 x *g* for 10 min at 4°C. The supernatant was removed and the pellet resuspended in an appropriate volume of DEPC treated water.

4.1.4 Determination of the integrity of RNA

The sample of RNA (5 µg) was prepared to a final volume of 10 µl with DEPC water and 1.7 µl of 6x RNA loading buffer. The samples (5 µg of RNA) were heated at 90°C for 2 min then rapidly transferred to ice for 2 min and microfuged before loading onto a formaldehyde agarose gel (see appendix). The RNA samples were electrophoresed for 1 hr at 70 volts. The integrity of the RNA was examined by visualisation of the 28s and 18s ribosomal RNA bands under UV light. If the 28 and 18s bands were sharp and clear then the mRNA was assumed to be intact.

4.1.5 Reverse transcription of RNA into 1st strand cDNA

Pancreatic tumour cell line RNA was reverse transcribed into first strand complementary deoxyribonucleic acid (cDNA) using the Superscript RNase H- Reverse Transcriptase kit (Gibco BRL U.K).

5.0 µg of total RNA and 500 ng of random hexamers in a total volume of 11 µl was incubated at 70°C for 10 min and then placed rapidly on ice. The contents of the tube were briefly (30 sec) centrifuged and 4 µl of 5x first strand buffer, 2 µl of 0.1 M DTT, 1 µl of mixed dNTP stock and 1.0 µl of superscript reverse transcriptase were added, vortexed to mix, microfuged and the mixture incubated at 37°C for 1 hr. Following transcription, the reverse transcriptase was heat inactivated at 70°C for 10 min. The cDNA was either stored at -20°C or immediately used for polymerase chain reaction (PCR).

4.1.6 CCK-R polymerase chain reaction

The CCK receptor primers were based on the CCK-A and CCK-B receptor sequences published by deWeerth *et al.* (1993a) and Pisegna *et al.* (1992a) and chosen using the oligo primer analysis software Rychlik *et al.* (1989) distributed by Medprobe (Oslo, Norway).

Two sets of gene specific primers were made and used to exclusively amplify a 340 base pair fragment from the CCK-A receptor and a 430 base pair fragment from the CCK-B receptor. Polymerase chain reaction (PCR) was performed on 2.0 µl of the first strand cDNA. Each 100 µl amplification reaction contained :- 200 µM of each dATP, dCTP, dGTP and dTTP (Gibco BRL, U.K); 1 µM of each cholecystokinin receptor specific primer; 10 µl of 10x Taq reaction buffer and 2.5 units of DNA polymerase (Promega, U.K). The cDNA was amplified for 35 cycles using 1 min at 94°C (denaturation), 1 min at 56°C (annealing) and 2 min at 72°C (elongation).

4.1.7 Electrophoresis of PCR products

The PCR products were electrophoresed on a 1% agarose gel containing ethidium bromide (0.3 µg/ml). 8.3 µl of 6x DNA loading buffer was added to 50 µl of the PCR product. The samples were loaded into each well of a 1% agarose gel, along with 0.5 µg of 1 kb DNA molecular weight marker (Gibco BRL) and electrophoresed at 70 volts for approximately 1 hr.

4.1.8 Gel Purification of PCR product

The PCR fragments were cleaned using the Qiaex II kit (Qiagen). The desired DNA bands were excised from the gel under UV light and placed in a preweighed eppendorf tube. The PCR products were exposed to a minimum amount of UV light in order to reduce nicking of the DNA. The weight of the agarose slice was determined. 3 volumes of buffer QX1 was added to 1 volume of gel and heated at 50°C for 5 min in order to allow the gel to melt. 10 µl of Qiaex beads were added to approximately 2 µg of DNA in the melted agarose. The agarose was heated at 50°C for 10 min with mixing every 2 min. The sample was then centrifuged at 6000 x *g* for 30 sec (pelleting the Qiaex beads) and the supernatant discarded. The pellet was washed with 500 µl of buffer QX2, by resuspending the beads in the buffer and centrifuging for 30 sec as above. Following this, the Qiaex beads were washed twice with 500 µl of buffer QX3 as above. After the second wash the pellet was air-dried for 15 min and resuspended in sterile water (approximately 10 µl) and incubated for 5 min at 50°C. The Qiaex bead suspensions were centrifuged at 6000 x *g* for 30 sec and the supernatant containing the DNA transferred to a clean tube. This step was repeated giving a final DNA volume of 20 µl. The DNA concentration of the sample was determined as described in the following section.

4.1.9 Spectrophotometric determination of DNA and RNA

Samples were diluted 1 in 100 with ddH_2O and absorbance was measured at 260 nm and 280 nm wavelengths. The absorption at 260 nm allows calculation of the concentration of nucleic acid in the sample whereas 280 nm is an indication of protein contamination. An OD_{260} of 1 is equivalent to approximately 50 µg/ml double stranded DNA or 40 µg/ml of RNA.

4.1.10 Purity of DNA and RNA

The ratio of OD_{260} over OD_{280} gives an approximation of the purity of the nucleic acid. The DNA and RNA are considered pure if the ratio is between 1.8 and 2.0. If the ratio is lower than 1.8 the sample could be contaminated with proteins and the quantitation of the amount of nucleic acid will prove difficult.

4.2 Cloning of the PCR amplified DNA

The CCK-A and CCK-B receptor PCR products were cloned using the TA cloning kit according to the manufacturers instructions (In vitrogen, UK) followed by plasmid DNA preparation as described below;

4.2.1 Ligation

Ligations were set up by incubating 2 µl of either the CCK-AR or CCK-BR PCR product in 5 µl of sterile water, 1 µl of 10x ligation buffer, 50ng pCR™ vector and 1 µl of T4 DNA ligase (1 unit) at 12°C overnight.

4.2.2 Transformation

One 50 µl vial of competent *E.coli* cells per ligation-transformation and a vial of 0.5 M ß-mercaptoethanol was thawed on ice. 2 µl of ß-mercaptoethanol was added to each vial of competent cells and mixed by gently tapping the tube. 1 µl of each ligated product was pipetted directly into the competent cells and mixed by gentle tapping. The vials were incubated on ice for 30 min followed by a 60 sec incubation at 42°C and rapidly placed on ice for a further 2 min. 450 µl of pre-warmed SOC medium was added to each vial of cells and incubated at 37°C for 1 hr with gentle agitation. Following the incubation, the transformed cells were placed on ice and 25 µl and 100 µl was spread on separate NZY agar plates (containing 40 mg/ml X-gal (Promega) and 50 µg/ml ampicillin). The plates were kept at room temperature for 5 min before inverting them and incubating at 37°C overnight.

4.2.3 Isolation of plasmid DNA-mini-preparation

White colonies were picked from the LB agar plates and grown for 10 hr at 37°C in L-broth containing 50 µg/ml ampicillin. Following their growth, 1.0 ml of the culture was transferred into eppendorf tubes and centrifuged at 1300 x *g* for 2 min, the pellet was resuspended in 100 µl of TE buffer, followed by lysis with 200 µl lysis buffer and placed on ice for 5 min. The lysate was treated with 150 µl of 3 M potassium acetate (pH 4.8) and centrifuged at 6000 x *g* for 10 min. The supernatant was transferred to a fresh eppendorf and extracted with an equal volume of phenol/chloroform (BDH). The

aqueous phase containing the DNA was precipitated with 2.5x volume of 100% ethanol at -20°C for 15 min. The DNA was pelleted, washed with 70% ethanol and resuspended in 30 µl of sterile water. The concentration of the DNA was determined by measuring the optical density reading at 260 nm.

4.2.4 Enzyme restriction of plasmid DNA

To determine the presence of the desired insert, a digest was set up as follows: 10 µl of plasmid DNA (from miniprep, approximately 1.0 µg), 4 µl of one phor all buffer (10x, Gibco BRL), 5 µl of water and 2 units of *Eco*RI (Gibco BRL) in a microcentrifuge tube. The digestion mix was briefly (30 sec) microfuged followed by incubation at 37°C for 1 hr. The digested products were electrophoresed on a 1% agarose gel. To each tube containing the *Eco*RI restricted plasmid 3 µl of 6x DNA loading buffer was added and the samples loaded onto the gel. The samples were electrophoresed at 70 volts for 1 hr, along with 0.5 µg of a 1 kb DNA molecular weight markers (Gibco BRL). The presence or absence of the DNA fragments was determined under UV light.

4.2.5 Isolation of plasmid DNA-maxi-preparation

500 ml of LB-broth containing 100 µg/ml ampicillin was inoculated with 10 ml of an overnight culture (harbouring the plasmid of interest) from above and allowed to grow at 37°C for 5 hr in a shaker at 400 rpm. The culture was centrifuged at 6000 x *g* for 15 min at 4°C. The pellet was resuspended in 5 ml of 25% sucrose in 50 mM Tris (pH 8.0) and placed on ice for 15 min. 0.5 ml of freshly prepared 20 mg/ml lysozyme in 0.25 M Tris (pH8.0) was added to the resuspended culture followed by the addition of 1.0 ml 0.5 M EDTA. The culture was gently mixed and held on ice for 10 min. 0.5 ml of 20% SDS was added, mixed gently, followed by the rapid addition of 2.5 ml of 5 M sodium chloride. The lysed cells were gently mixed and incubated on ice for 1 hr. The lysate was centrifuged at 20,000 x *g* for 1 hr at 4°C. The supernatant was transferred to 50 ml falcon tubes and the volume made up to 25 ml with TE. 10 ml of phenol followed by 10 ml of chloroform was added to the supernatant, which were mixed well and centrifuged at 560 x *g* for 15 min. The upper (aqueous layer) was re-extracted with

25 ml of chloroform (560 x *g*, 10 min). Following the second organic extraction, the aqueous layer was transferred to a fresh tube and 0.6 volumes of isopropanol was added, the precipitated DNA was recovered by centrifuging the mixture at 15,000 x *g* for 10 min. The supernatant was discarded and the pellet dried (by inverting the tube on a tissue for 10 min). The dried pellet was resuspended in an exact volume of 9.8 ml TE containing 40 µg/ml RNAse (4 µl of 10 mg/ml) and incubated at 37°C for 30 min. The plasmid DNA was further purified by centrifugation in caesium chloride-ethidium bromide density gradients.

4.2.6 Caesium chloride-ethidium bromide equilibrium centrifugation

Caesium chloride (9.8 g) was added to 9.8 ml of TE buffer (containing the DNA) and the resulting mixture was loaded into quick seal ultracentrifuge tubes, prior to layering with 200 µl of 10 mg/ml ethidium bromide. The tubes were sealed, mixed by inversion and placed in a Beckman Vti65 rotor and ultracentrifuged for 24 hr at 350,000 x *g* at 20°C. The top of the tube was then cut off and the plasmid DNA (visible as a thin band) was recovered by suction using an 18GA needle. The DNA was transferred to a 50 ml falcon tube topped up to 0.5 ml with TE buffer and extracted with isopropanol saturated caesium chloride. The tube contents were mixed thoroughly and centrifuged at 360 x *g* for 5 min at room temperature. The upper pink organic layer was discarded and the extractions repeated until the organic layer became colourless. To precipitate the DNA the sample was then diluted with 2 volumes of water and 6 volumes of cold (4°C) ethanol, mixed and incubated at 4°C for 30 min followed by centrifugation at 18, 000 x *g* for 30 min at 4°C. The supernatant was discarded and the pellet was washed several times to remove any traces of caesium chloride in 70% ethanol. Once the pellet was washed, it was dissolved in sterile water and the DNA concentration determined by measuring the optical density at UV wavelength 260 nm.

4.3 DNA sequencing

The DNA was sequenced using the Pharmacia sequencing kit (appendix I, p286) as described below;

4.3.1 Annealing of primer to double stranded template

8 µl of 2 M sodium hydroxide was added to 1.5 µg of DNA template (in a volume of 32 µl) and incubated at room temperature for 10 min. 7 µl of 3 M sodium acetate (pH 4.8) was added followed by 120 µl of 100% ethanol; the DNA was precipitated at -20°C for 15 min followed by centrifuging at 6000 x *g* for 15 min at 4°C. The pellet was washed with 70% ethanol and resuspended in 7 µl of water. 10 pmol of primer (2µl, universal primer), 2 µl of annealing buffer and 3 µl of DMSO were added to the 7 µl of the denatured DNA template, mixed and incubated at 37°C for 20 min. Following this, the annealing mixture was placed at room temperature for 10 min before proceeding with the labelling reaction.

4.3.2 Labelling reaction

To the tube containing the annealed primer and DNA template, 3 µl of labelling mix A (see appendix I, p286), 5 µCi of ^{35}S-dATP, and 2 µl (3.2 units) of T7 DNA polymerase was added, mixed and incubated at room temperature for 5 min. For each template to be sequenced 2.5 µl of each short mix of G, A, T and C were pipetted into eppendorfs and prewarmed at 37°C.

4.3.3 Termination reaction

Following the labelling reaction, 4.5 µl of this mix was added to 2.5 µl of the prewarmed short mix and incubated for a further 5 min at 37°C. The reaction was terminated by the addition of 5 µl of stop buffer. The terminated reactions were either electrophoresed immediately or stored at -20°C.

4.3.4 Preparation of PAGE sequencing gels

For each gel, a pair of front and back glass gel plates were washed meticulously with Hibiscrub and water. The plates were rinsed well with ddH_2O and dried with lint-free

paper towel and then wiped with 70% ethanol. A film of 5% dimethyldichlorosilane in chloroform was carefully applied to one of the plates in a fume cupboard with a Kimwipe soft tissue. Once the film dried, the plate was wiped with 70% ethanol and dried. The plates were assembled according to the manufacturer's instructions with 0.4 mm uniform thickness spacers and clamps. For each gel, 50 ml of desired acrylamide gel solution (see appendix I for details) in a 100 ml beaker was prepared. The solution was thoroughly mixed with 200 µl of 20% ammonium persulphate prior to pouring. The acrylamide solution was gently pulled into a 60 ml syringe avoiding any air bubbles. The gel apparatus was raised to a 45° angle from the bench top (with the short plate on top) and the acrylamide solution slowly delivered between the plates down one side. When the solution reached the top of the short plate, the gel sandwich was positioned with the top edge 5 cm above the benchtop. The flat side of a 0.4 mm sharktooth comb was inserted 2 to 3 mm into the solution below the top of the short plate avoiding air bubbles and extra acrylamide solution was layered onto the comb to ensure full coverage. The gel was allowed to polymerise overnight before use.

4.3.5 Electrophoresis of sequencing gels

The comb was removed gently from the set gel sandwich, avoiding pulling or stretching the top of the gel. The bottom reservoir of each gel apparatus was filled with 1x TBE buffer such that the gels would be submerged 2 - 3 cm in buffer. Each gel sandwich was then placed in a sequencing electrophoresis apparatus and plates clamped in position. 1x TBE buffer was poured between the plates, and the top of the gel rinsed by syringe with TBE buffer. Each gel was pre-heated at a constant power of 60 W for approximately 30 min before loading the sequencing samples. The comb was inserted into the pre-heated gel and any bubbles removed by syringe. The labelled reactions were heated at 70°C for 2 min and 3 µl loaded per well on each gel to be run. The samples were electrophoresed for approximately 2.5 hr at 50 W.

4.3.6 Fixing of gels

The buffer was drained from the sequencing apparatus following gel electrophoresis and the gel sandwich dismantled by removing the clamps and carefully separating the smaller plate from the larger plate. Once separated, the spacers were removed and the

plate with the gel was placed horizontally in a shallow tray. The gel was gently covered to a depth of 2 cm with 10% acetic acid/10% ethanol fixer solution for 15 min. The fixer solution was then discarded and the gel plate removed to a benchtop. Two pieces of dry blotting paper cut to the gel size were placed together on top of the gel, beginning at one end of the gel and working slowly towards the other to prevent bubbles forming between the paper and the gel. The blotting paper was then peeled off the plate with the gel and covered with saran wrap and placed on a preheated gel dryer for 15 min at 80°C. Once dried the saran wrap was removed and the gel placed in an X-ray cassette containing Kodak XAR-5 film. Following an overnight (16 hr) exposure at room temperature the X-ray film was removed and processed.

4.4 Determination of CCK-R expression in human pancreatic cancer cell lines by RNase protection assay

The RNase protection assays were carried out using the RPA II kit purchased from AMS Biotechnology (UK, see appendix I, p287).

4.4.1 Construction of DNA templates

The plasmids containing the DNA fragments (described in sections 4.2.5-4.2.6) were digested with the appropriate restriction enzymes to enable synthesis of anti-sense and sense RNA probes. The plasmid DNA (100 µg) was digested in 100 µl total volume as follows: 10 µl of 10x digestion buffer, 2 µl of enzyme, x µl of plasmid DNA (100 µg) and sterile water added to make the 100 µl volume. *Bam*HI (Promega, UK) in buffer E was used to construct templates for the synthesis of an antisense CCK-AR and sense CCK-BR RNA probe. Templates for the synthesis of an antisense CCK-BR and sense CCK-AR RNA probe were constructed using *Ava*I (Promega, UK) in buffer B. The digestion was allowed to proceed at 37°C overnight (16 hr) followed by DNA purification the following day: An equal volume of phenol/chloroform (1:1) was added to the digestion reaction and the samples vortexed and microfuged for 2 min at 6000 x *g* in an eppendorf tube. The upper aqueous layer was transferred to a fresh tube and the extraction repeated with phenol/chloroform. The aqueous layer was re-extracted with chloroform (100 µl), vortexed and microfuged for 2 min. 2.5x the volume of 100% ethanol was added to the aqueous layer and incubated at -20°C for 15 min and

microfuged for 10 min as above. The supernatant was decanted and the pellet washed in 70% ethanol and air dried prior to dissolving in water. UV absorbance at 260 nm wavelength was measured and the concentration of DNA calculated. The sense RNA probe being an internal control for the RNase protection assay.

4.4.2 Synthesis of high specific activity radiolabelled RNA probes

The RNA probes were synthesised using the AMS biotechnology (UK) maxi-script kit. Briefly 1.0 µg DNA template was transcribed as follows: all solutions were allowed to reach room temperature before use.

Nuclease free water	4 µl
Transcription buffer (10x)	2 µl
200 mM DTT	1 µl
ATP, GTP and UTP (2.5 mM each) prepared by mixing 1 volume of water with one volume of each 10 mM ATP, GTP and UTP stocks supplied)	4 µl
rRNasin ribonuclease inhibitor (20 U)	1 µl
Linearised DNA template (1.0 µg/µl)	1 µl
100 µM CTP	1 µl
[α ^{32}P] CTP (10 mCi/ml)	5 µl
SP6 polymerase (CCKB) or T7 polymerase (CCKA) (20 U)	1 µl
Total volume	20 µl

The above reaction was incubated at 37°C for 60 min: following this, 2 units of DNase I was added and further incubated for 15 min to remove the template DNA. The reaction was terminated by the addition of 21 µl of sample buffer and the labelled transcript was purified by polyacrylamide gel electrophoresis (4.4.3). This resultant probe had a specific activity of approximately 4 x 10^8 cpm/µg. An internal GAPDH RNA probe was also synthesised using GAPDH (glyceraldehyde-phosphate-dehydrogenase) DNA template (AMS Biotechnology) and T3 polymerase.

4.4.3 Gel purification of ^{32}P-labelled RNA probe

2 µl of the terminated reaction was transferred into a fresh eppendorf tube containing 198 µl of TE buffer with 100 µg of carrier RNA (yeast RNA) and the remaining sample was treated at 95°C for 4 min and loaded onto a 6% polyacrylamide gel (see 4.3.4). The probe was electrophoresed for 3 hr and the gel covered with saran wrap and exposed to X-ray film for approximately 2 min. During exposure, a designated corner of the film was marked for orientation and lines were drawn with a felt-tip pen across the corners and sides of the film. The film was developed and used to precisely localise the area of the gel containing the full-length labelled transcript. The film was aligned with the gel and the area of the gel containing the probe was excised with a scalpel and transferred with clean forceps to a microfuge tube containing 350 µl of elution buffer. The elution of the probe was carried out overnight (16 hr) at room temperature. The following day the gel was removed from the elution buffer and 1 µl of the elution buffer containing the probe was transferred into 3 ml of scintillation fluid and the radioactivity measured in a Packard 2000CA counter.

4.4.4 Calculation of probe specific activity

100 µl of the probe diluted in TE buffer (containing yeast RNA) was counted directly in 3 ml of scintillation fluid (total count). The amount of ^{32}P incorporated into the RNA probe was determined by trichloroacetic acid (TCA) precipitation. The remaining 100 µl of the diluted reaction was added to 2 ml of cold 10% TCA, vortexed and held on ice for 5 min. The precipitate was collected by filtering under vacuum on GF/C glass fibre filters and then was washed once with 2 ml of cold 10% TCA and twice with 95% ethanol. The filter was oven dried and transferred into scintillant (3 ml) for counting (Packard 2000CA counter). Calculation of the specific activity of the probe;

Proportion of ^{32}P-CTP incorporated into RNA:

$$\frac{1.3 \times 10^6 \text{ cpm TCA}}{2.6 \times 10^6 \text{ cpm total}} \times 100 = 50\ \%$$

Moles of CTP in reaction:

$$\text{No of mCi } {}^{32}\text{P in reaction} = 5\ \mu l \times \frac{10\ mCi}{ml} \times \frac{1\ ml}{1000\ \mu l} = 0.05\ mCi$$

$$\text{No of mmol } {}^{32}\text{P-CTP} = 0.05\ mCi \times \frac{1\ mmol}{400\ Ci} \times \frac{1\ Ci}{1000\ mCi} = \frac{0.05}{4 \times 10^5}\ mmol$$

$$0.0125 \times 10^{-5}\ mmol = 0.125\ nmol \text{ in reaction}$$

No of moles of cold CTP:

$$\frac{100\ \mu mol}{1000\ ml} \times 1\ \mu l \times \frac{1\ ml}{1000\ \mu l} = 100 \times 10^{-6} = 0.1\ nmol$$

Total moles of CTP = 0.125 nmol of ^{32}P- CTP + 0.1 nmol cold CTP = 0.225 nmoles

Total CTP incorporated into RNA:

0.225 nmol in reaction x 50% incorporation = 0.1125 nmol incorporated

$$\text{RNA synthesised in nanogrammes} = \frac{1320 \times 10^9\ ng}{10^9\ nmol} \times 0.1125\ nmoles$$

$$= 148.5\ ng$$

cpm incorporated into the RNA product:

$42\ \mu l \times 1.3 \times 10^6\ cpm/\mu l = 54.6 \times 10^6$ cpm of TCA-precipitable material (*i.e.* RNA)

Specific activity of the product:

$$\frac{54.6 \times 10^6\ cpm}{148.5\ ng} \times 1000 = 3.68 \times 10^8\ cpm/\mu g$$

4.4.5 Hybridisation of probe and sample

RNA (50 µg) from each cell line and positive control samples was hybridised with 600 pg of radioactively labelled RNA probe at 42°C for 12 hr in 20 µl of hybridisation buffer. Following hybridisation 200 µl of digestion buffer containing 1.0 µg of RNase, was added to the hybridised samples and incubated at 37°C for 30 min to degrade any remaining single-stranded RNA. The protected RNA was precipitated at -20°C for 30 min with 300 µl of solution Dx and centrifuged for 30 min at 6000 x *g*. The pellet was resuspended in 8 µl of RNA loading buffer and then treated at 95°C for 4 min prior to electrophoresis on a 6% polyacrylamide gel for 3-5 hr at 50 W.

Following electrophoresis, the buffer was drained from the gel apparatus and the gel sandwich dismantled by separating the plates carefully. The gel was fixed and dried as in section 4.3.6. Kodak XAR-5 film was exposed to the gel in an X-ray cassette at -70°C in the presence of an intensifying screen. Five days later the X-ray film was removed and processed.

4.5 Radioligand binding studies to determine the presence of CCK-BR in Mia PaCa-2 cells

4.5.1 Preparation of cells

The cell lines Mia PaCa-2 and NIH3T3CCK-BR were grown in culture as described in 2.1. The cells were treated with versene (Sigma) rather than trypsin in these experiments. 1 x 10^9 cells were harvested with versene and centrifuged at 800 x *g* for 10 min. The cells were resuspended in Hepes-NaOH buffer (pH 7.2 at 21°C) to give a concentration of 1 x10^9 cells per 10 ml.

4.5.2 Incubation conditions

The cells were aliquoted at various concentrations, (3 x 10^7, 1 x 10^7, 3 x 10^6, 1 x 10^6, 3 x 10^5, 1 x 10^5, 3 x 10^4) in a 400 µl volume and incubated with 50 µl of 1.0 nM [^{3}H]-PD140,376, for 150 min at 21°C. Total and non-specific binding of [^{3}H]-

PD140,376 (a specific CCK-BR antagonist) were defined in the absence and presence of 50 µl of 10 µM L-365,260 (a specific CCK-AR antagonist) respectively. The assay was terminated by rapid filtration through presoaked Whatman GF/B filters which were washed (3 x3 ml) with 50 mM Tris HCl (pH 7.4 at 4°C) using a Brandell cell harvester. The filters were transferred into scintillation vials containing 5 ml Meridian Goldstar liquid scintillation cocktail. The bound radioactivity was determined after 4 hr by counting (3 min) in a Beckman LS6500 liquid scintillation counter.

4.6 Analysis of K-*ras* status

4.6.1 Isolation of genomic DNA from cell lines

The cell lines were grown in the appropriate culture medium to 70% confluency. Genomic DNA from the cell lines was prepared as follows: Cells from culture flasks were harvested with trypsin, centrifuged (as mentioned previously) and lysed at 55°C for 24 hr with 1 ml TNE containing 0.66% SDS and 1.5 ng proteinase K. Following the digestion the cell lysate was extracted with an equal volume of phenol for 20 min and centrifuged at 440 x *g* for 10 min to separate the two phases. The aqueous phase was transferred to a fresh eppendorf and re-extracted with phenol for 20 min followed by an extraction with chloroform. Following separation of the two phases the bottom organic (chloroform) layer was removed and discarded appropriately. The aqueous DNA phase was treated with 0.1 µg of RNase at room temperature for 20 min. The DNA phase was re-extracted with an equal volume of phenol. The upper aqueous phase was transferred to a fresh eppendorf tube and the DNA precipitated with 2-3 volumes of chilled absolute alcohol at 4°C for 30 min. The tube was gently inverted a few times and the precipitated DNA removed with a clean tip and rinsed in 70% ethanol. The DNA was left to air dry and finally dissolved in a small volume of sterile water. The DNA concentration was determined as described in 4.1.9.

4.6.2 K-*ras* polymerase chain reaction

The sequence of the K-*ras* primers was obtained from the work of Caldas *et al.* (1994) and Sumi *et al.* (1994) for exons 1 and 2 respectively. The primers were synthesised by Oswell (Southampton, U.K, see appendix I, p282)

4.6.2.1 PCR amplification of exon 1

50 ng of genomic DNA from each pancreatic tumour cell line was amplified by PCR. Each 20 µl amplification reaction contained; 200 µM of each dATP, dCTP, dGTP and dTTP (Gibco BRL, U.K); 1 µM of each primer; 2 µl of 10x Taq reaction buffer and 0.5 units of DNA polymerase (Promega, U.K). The genomic DNA was amplified for 35 cycles using 30 s at 95°C (denaturation), 30 s at 58°C (annealing) and 30 s at 72°C (elongation).

4.6.2.2 PCR amplification of exon 2

50 ng of genomic DNA was amplified as above for 30 cycles using 1 min at 96°C (denaturation), 1 min at 56°C (annealing) and 1 min at 74°C (elongation) followed by a time delay at 74°C for 7 min.

The PCR products were electrophoresed on a 1.8% agarose gel and stained with ethidium bromide. The PCR product was gel purified and cloned using the Qiaex II kit (section 4.1.8) and TA cloning kit (section 4.2), respectively.

4.6.3 T7/T3 PCR to determine the presence of K-*ras* PCR product

PCR was performed on the selected colonies. A colony was selected by a pipette tip and dipped into the PCR mix and then this same tip with the colony remains was transferred to a snap-top tube containing NZY broth with 50 µg/ml ampicillin. Each 20 µl amplification reaction contained :- 200 µM of each dATP, dCTP, dGTP and dTTP; 1 µM of each primer, T3 and T7; 2 µl of 10x Taq reaction buffer and 0.5 units of DNA polymerase (Promega, U.K). The DNA was amplified for 30 cycles using 1 min at 94°C (denaturation), 1 min at 55°C (annealing) and 3 min at 72°C (elongation). The PCR products were electrophoresed on a 1.8% agarose gel containing ethidium bromide (0.1 µg/ml).

The presence of the desired fragment was determined and plasmid DNA isolated (mini-preparation in 4.2.3).

4.6.4 PCR-Single strand conformational polymorphism (SSCP)

PCR of K-*ras* exon 1 was carried out described in 4.6.2.1. but in the presence of 2 µCi of 3000 Ci/mmol deoxy-cytidine triphosphate (dCTP) (Amersham). Following amplification, 10 µl of the PCR product was diluted with 125 µl of 10 mM EDTA containing 0.1%SDS. 4 µl of loading buffer was added to 4 µl of the diluted PCR product and the sample treated at 95°C for 5 min, rapidly cooled on ice and electrophoresed on a 6% polyacrylamide gel containing 10% glycerol at 5 W for 14 hr in 1x TBE. The gel was transferred to 3MM Whatman paper and exposed to Kodak film for 24 hr at -70°C.

4.7 Semi-quantitative CCK-R RT-PCR

RNA was extracted from pancreatic tumours along with their matched normal counterparts as described in section 4.1.3 and the RNA integrity determined as in section 4.1.4. cDNA was synthesised from 5 µg of total RNA (section 4.1.5). Semi quantitative PCR was carried out as follows: PCR was performed on 1.0 µl of the neat or diluted cDNA. The cDNA was diluted 1:10, 1:100, 1:1000 and 1:10,000 fold with sterile water. Each 50 µl amplification reaction contained :- 200 µM of each dATP, dCTP, dGTP and dTTP (Gibco BRL, U.K); 1 µM of each cholecystokinin receptor specific primer; 0.5 µM of each ß-actin primer; 10 µl of 10x Taq reaction buffer and 2.5 units of DNA polymerase (Promega, U.K). The cDNA was amplified for 35 cycles using 1 min at 94°C (denaturation), 1 min at 56°C (annealing) and 2 min at 72°C (elongation). The PCR products were electrophoresed on a 1% agarose gel containing ethidium bromide (0.1 µg/ml).

CHAPTER 5

IN VIVO XENOGRAFT STUDIES

CHAPTER 5

5.1 Animals

The immunodeficient mice were purchased from Charles River Ltd. (UK). Male CD-1 nude and CB-17 SCID mice were purchased at 5 weeks of age. The mice were housed in sterile plastic flexible film isolators (Isotech Ltd., UK) with autoclaved bedding, food and water. They were acclimatised to a 12 hr light/dark cycle. The temperature and humidity in the isolator were maintained at 23°C and 40-55%, respectively. Mice were housed 3 per cage.

5.2 Xenografting of primary pancreatic tumours

Fresh pancreatic cancer specimens obtained from theatre were removed to a class II cabinet and immediately minced with the aid of a scalpel in tyrode solution (Gibco BRL) at room temperature and the resultant suspension of cells taken up into a syringe with a 23 gauge needle. The syringe and needle were wiped with virkon (sterilising solution) before passing into the isolator. The male CD-1 nude mice were injected (0.1-0.3 ml) subcutaneously into the left flank and monitored daily.

0.1-0.3 ml of the tumour suspension was also grown *in vitro* in 10% FCS in DMEM/Ham's F12 with sodium bicarbonate supplemented with 15 mM HEPES, 16 nM epidermal growth factor, 1.4 μM hydrocortisone, 1 μM insulin, 5.0 μM isobutylmethylxanthine, 5 mM glutamine and 100 U/ml penicillin and streptomycin in a sterile incubator (Leec) at 37°C with 10% carbon dioxide. When the cells reached 70% confluency (between 7-12 days) tissue culture medium was decanted and 4 ml of trypsin added to the cells for 5 min at room temperature. Once the cells were detached from the petri-dish the above medium was mixed into the trypsinised cells. A sample of the cells were counted using trypan blue (see section 2.2) and the remaining cells replated in a petri-dish at 5×10^4 in fresh medium. This procedure was repeated as required.

5.2.1 Passaging of xenografted tumours

When the xenografted tumours reached approximately 10% of the mouse body mass, the mouse was killed by ether inhalation followed by cervical dislocation and the tumour removed utilising aseptic techniques. The tumour was trimmed of any adhering fatty tissue, washed with tyrode and blood removed prior to mincing into a suspension with a scalpel and a pair of forceps in 5 ml of tyrode solution. The cell suspension was taken up into a 1.0 ml syringe and the cells injected subcutaneously through a 23 gauge needle into the left flank of a nude mouse. The maximum volume of cell suspension injected was 0.3 ml. The mice were monitored daily and when the tumour approached 10% of the body mass the above procedure was repeated.

5.3 Extraction of DNA from xenografts

The tumour (approximately 300 mg) was homogenised (60 sec) using a Silverson Polytron tissue grinder in 900 µl of 1x TNE at room temperature. Following this 1.5 ng of proteinase K and 0.66% SDS was added to the ground tissue (1.0 ml). The tissue was incubated overnight at 55°C. The following day DNA was extracted from the tissue as described in 4.6.1 and the concentration determined as in section 4.1.9.

5.4 CCK-R and K-*ras* status in the passaged tumours

RNA was extracted from the original and passaged tumour as described in section 4.1.3. The RNA was reverse transcribed (section 4.1.5) and the cDNA subjected to CCK-R (section 4.1.6) and K-*ras* amplification (sections 4.6.2.1-2). The K-*ras* PCR products were cloned and sequenced (sections 4.2.1-3 and 4.3 respectively).

5.5 Cell culture

The cell lines (Mia PaCa-2, Panc-1, BxPc-3 and Hs766T) were grown as described in 2.1. When sufficient number of cells were obtained the cells were centrifuged as above and resuspended in serum-free DMEM at a cell density of 2. 5 x 10^7 cells per 1.0 ml.

5.5.1 Xenografting of human pancreatic cancer cell lines

The vial containing the cells was wiped with virkon before passing into the isolator. Prior to injecting the cells the mice were randomly grouped into cages of 3. 0.2 ml of the cells (5 x10^6 cells) were injected subcutaneously into the left flank of the male CD-1 nude mice and CB-17 SCID mice using a 19 gauge needle. Each mouse was individually marked for identification purposes. The tumour was allowed to grow to a maximum of 10% of the mouse body mass.

5.5.2 Agonist and Antagonist studies

The Mia PaCa-2 cells were cultured and injected on day 0 into male CD-1 nude mice as described in section 5.5.1. On day 15 the presence of palpable and visible tumours (approximately 2 mm x 2 mm) was determined. The mice were weighed every fortnight from this point onwards to the end of the study. All drugs were injected subcutaneously into the scruff of the neck in 0.1 ml of the appropriate vehicle. The mice were injected on a daily basis with appropriate doses of sCCK-8, nsG-17 (15 µg/kg, 30 µg/kg and 100 µg/kg), or 1 mg/kg of the various antagonists, 20 mg/kg of JB93182 or vehicle controls. The mice were treated for 49 days and then sacrificed on day 64.

5.5.3 Autopsy procedures and sample collection

The pancreatic tumours and spleens were removed carefully, trimmed of adhering fatty tissue and weighed. A specimen of the tumour was stored in 10% formalin and the remaining tumour and whole spleen were snap frozen and stored in liquid nitrogen. Each mouse was macroscopically examined for any visible abnormalities as well as any metastasis to the abdomen, lungs and axilla.

5.5.4 Determination of protein and DNA content

The tumour specimens from the agonist/antagonist studies were divided in two for storage in liquid nitrogen. Approximately 300 mg of tumour was used for DNA extraction and the remainder used for protein extraction. The DNA and protein contents were determined as described in sections 5.3 and 3.3 respectively.

CHAPTER 6

ELUCIDATION OF NOVEL PROTEIN KINASES IN PANCREATIC CANCER

CHAPTER 6

6.1 Protein kinase PCR

All buffers, kits, chemicals and solutions used are referred to in appendix I, p288-290. PCR was performed on 2.0 µl of the first strand cDNA synthesised from total RNA extracted from 2 human pancreatic cancers, PT1 and PT3 as described in 4.1.3 and 4.1.5. Each 100 µl amplification reaction contained :- 200 µM of each dATP, dCTP, dGTP and dTTP (Gibco BRL); 20 pM of each primer PTKI and PTKII, 10 µl of 10x Taq reaction buffer and 2.0 units of DNA polymerase (Promega, U.K). The cDNA was amplified for 30 cycles using 90 sec at 93°C (denaturation), 2 min at 45°C (annealing) and 4 min at 72°C (elongation).

6.2 Gel electrophoresis of the PCR product

The PCR products were electrophoresed on a 1.8% low melting point agarose gel (15 x 13 cm) containing 0.1 µg/ml ethidium bromide. The samples were loaded into each well along with 0.5 µg of 1 kb DNA molecular weight markers (Gibco BRL) and electrophoresed at 70 volts for approximately 1 hr. The DNA was visualised under UV light.

6.3 Phenol/chloroform purification of the PCR product

The PCR product (220 base pairs) was excised from the gel using a sharp blade and placed in a preweighed eppendorf tube. The excised gel weight was determined and then melted at 70°C. To the melted agarose, 10% of the total volume of 10x TNE was added. The agarose was extracted with equal volume of water saturated phenol (BDH), vortexed and microfuged at 6000 x *g* for 2 min. The upper aqueous layer was transferred to a fresh eppendorf tube and the remaining bottom organic layer was re-extracted with an equal volume of 1x TNE. The phenol phase was discarded. The upper layer was pooled into the clean eppendorf following microcentrifugation and extracted with an equal volume of phenol/chloroform, vortexed and microfuged as above. The upper aqueous layer was extracted with an equal volume of chloroform (BDH), vortexed and microfuged at 6000 x *g* for 2 min. The lower organic layer was removed and discarded. To the remaining aqueous layer 2.5x volumes of 100%

ethanol was added and the sample stored at -20°C for 30 min followed by a 15 min centrifugation at 6000 x *g*. The supernatant was decanted and the pellet washed in 70% ethanol. The washed pellet containing the 220 bp PCR fragment was air-dried and dissolved in 35 µl of sterile water.

6.4 Enzyme restriction of the purified PCR products

The 220 bp PCR fragment was digested by the enzymes *ECo*R1 (Gibco-BRL) and *XhO*1 (Gibco-BRL) as follows: 35 µl of DNA (clean PCR product from above), 4 µl of enzyme restriction buffer D, 1 unit of *ECo*R1 and 2 units of *XhO*I. The digestion was carried out at 37°C for 2 hr. The digested product was electrophoresed on a 1.8% agarose gel (15 x 13 cm) as in 6.2.

6.5 Gel purification of the digested PCR product

The digest was purified using the Qiaex II kit (4.1.8) and dissolved in a final volume of 20 µl. The concentration and purity of the DNA in suspension was calculated having measured the OD at UV wavelength 260 nm (4.1.10).

6.6 Ligation of enzyme-restricted PCR products into phage vector

The digest was ligated into the phage vector using the ZAP express™ predigested vector kit from Stratagene. The ligation reaction was set up in a final volume of 20 µl as follows; 1 µg (1 µl) of phage arms, 15 µl of diluted insert (DNA from above), 2 µl of 10x ligase buffer and 8 units of T4 DNA ligase (1 µl). The ligation was carried out on ice overnight.

6.7 Packaging of ligated DNA into lambda vector

Packaging of ligated DNA was performed using the Packagene lambda DNA packaging system from Promega. The packagene extract (50 µl) was thawed on ice and 0.5 µg (10 µl) of the ligated DNA fragment added. This extract was mixed by gentle tapping of the eppendorf and then incubated at room temperature (22°C) for 3 hr. Following this, phage buffer (445 µl) and chloroform (25 µl) was added to the 60 µl packaging

mix and gently agitated allowing the chloroform to settle at the bottom of the eppendorf tube. The packaged phage was now stored at 4°C if not used immediately.

6.8 Preparation of bacterial culture for infection

E.Coli cells (XL1) were grown overnight at 37°C in NZY broth containing 0.2% maltose and 10 mM magnesium sulphate. The following day OD readings at 600 nm wavelength were measured against water. The required OD of 0.5 was obtained by altering the density of the bacterial cells in 10 mM magnesium sulphate (*i.e.* cells were pelleted and resuspended in an appropriate volume of 10 mM magnesium sulphate).

6.9 Titration of the packaged phage on NZY plates

200 µl of the above diluted bacterial cells were incubated with i) 2 µl and ii) 10 µl of the packaged phage stock at 37°C for 15 min. Meanwhile NZY agar plates were placed in a 37°C oven to pre-warm and top agar was melted in a microwave and incubated at 48°C once molten. To 3 ml of the top agar 15 µl of 0.5 M IPTG and 50 µl of 250 mg/ml X-gal was added. This was rapidly added to the cocktail of bacterial cells and packaged phage following the 15 min incubation and then mixed and poured onto the NZY agar plates. The agar was allowed to set at room temperature and the plates were incubated at 37°C overnight. The following day the plaques were counted. (NB. If no plaques were obtained then further titrations of the packaged phage would have to be carried out until plaques were formed).

Day 1: Once a sufficient number of plaques were obtained, they were cored out from the NZY agar plates with the aid of a Pasteur pipette and transferred into 500 µl of SM buffer containing 20 µl of chloroform. This extract was vibrated for 2 hr (allowing the phage to diffuse into the solution) resulting in the phage stock. The XL1 bacterial cells were grown overnight in NZY broth at 30°C.

Day 2: Following the overnight incubation, the XL1 cells were diluted 1:50 and grown for a further 4 hr in NZY broth containing 10 mM magnesium sulphate and 0.2% (w/v) maltose at 37°C. They were then resuspended in 10 mM magnesium sulphate to give

an OD of 1.0. To 200 µl of these XL1 cells 1 µl of EXAssist helper phage and 250 µl of the phage stock was added. This cocktail was incubated at 37°C for 15 min and transferred to 3 ml NZY broth followed by an overnight incubation in a shaker at 37°C. NZY agar plates containing kanamycin at a concentration of 50 mg/l were prepared for the following day. XLOLR (*E.coli* strain) bacterial cells were also grown overnight at 37°C in NZY broth for use the following day.

Day 3: The overnight culture of XLOLR cells was diluted 1:100 into NZY broth and grown for a further 4 hr at 37°C in a shaker. The cultures containing the phage stock were treated at 70°C for 15 min then centrifuged at 4000 x *g* for 15 min. The supernatant containing the filamentous phage stock was transferred to a fresh tube and stored at 4°C until used. 4 hr later the XLOLR cells were pelleted and resuspended to an OD of 1.0 in NZY broth. 200 µl of the resuspended XLOLR cells were transferred into eppendorfs to which 5 µl of the filamentous phage stock was added. The cocktail was incubated at 37°C for 15 min and spread onto NZY plates containing kanamycin. The plates were incubated overnight at 37°C in a humid incubator.

Day 4: A colony was selected from each plate and grown overnight in NZY broth containing kanamycin at 37°C in a shaker.

Day 5: Following the overnight growth DNA was extracted from the cultures by the mini-preparation method and sequenced as in sections 4.2.3 and 4.3 respectively. Once the sequence of each kinase extracted was determined bacterial stocks were prepared for each different kinase.

6.10 Freezing of bacterial stocks

1.0 ml of bacterial culture with the appropriate kinase DNA insert was centrifuged at 1300 x *g* for 2 min. The resultant supernatant was discarded and the pellet resuspended in 1.5 ml of 50% glycerol (Sigma) in NZY broth. The stock was stored at -70°C for use at a later date.

6.11 Determination of isolated protein kinases in human pancreatic cancers and cell lines.

6.11.1 Protein kinase PCR

Total RNA was extracted from 4 human pancreatic specimens (PT1, PT2, PT3 and PT4), 3 normal pancreas tissues (PN1, PN2 and PN3) and 4 human pancreatic cancer cell lines (Mia PaCa-2, Capan-1, Hs766T and BxPc-3) as mentioned in sections 4.1.3 and 4.1.2 respectively. First strand cDNA was reverse-transcribed as in section 4.1.5 using 0.1 µg of oligo dTTT rather than random hexamers.

The kinase PCR was carried out as in section 6.1 in a final volume of 20 µl using 1 µl of the prepared cDNA. To 20 µl of the PCR product 3.3 µl of DNA loading buffer was added and the sample electrophoresed on a 1.8% agarose gel at 70 volts for approximately 1 hr. The band corresponding to the 220 bp PCR product was cut out and cleaned using the Qiaex II kit (4.1.8). The DNA was eluted into a final volume of 18 µl.

6.11.2 Synthesis of ^{32}P labelled DNA probe

6 µl of the cleaned PCR product DNA from above was denatured at 99°C for 10 min mixed with 5 µl of OLB (5x) and 5 µl of sterile ddH_2O and rapidly placed on ice for 2 min. 1 µl of 10 mg/ml bovine serum albumin (BSA), 1 unit of Klenow and 20 µCi of $\alpha^{32}P$-dCTP (DuPont) was added to the denatured DNA, vortexed briefly, centrifuged and incubated at room temperature for 1 hr. The DNA was denatured again after the 1 hr incubation at 99°C for 2 min, rapidly placed on ice and held for 2 min followed by the addition of 1 unit of Klenow. The mixture was briefly vortexed, centrifuged and incubated for a further 1 hr at room temperature. The labelling reaction was terminated by the addition of 70 µl of sterile water.

The unincorporated nucleotides were removed by passing the labelled reaction through sephadex G50. This was carried out by centrifuging 1 ml of sephadex G50 in spin-X centrifuge filter columns (Costar) at 6000 x *g* for 30 sec. The eluate was discarded and

the step above repeated with a further 500 µl of sephadex G50. The labelled reaction was applied to the sephadex G50 and the sample centrifuged at 6000 x *g* for 30 sec. The upper filter unit containing the sephadex plus unincorporated nucleotides was disposed of appropriately and the eluate containing the radioactive probe stored at -70°C (if not used immediately).

6.11.3 Dot blotting of DNA

A dot blot with the DNA from each kinase elucidated was set up using a hybrid dot manifold apparatus (Bethesda research Labs, USA). Some preliminary work was required prior to the manifold assembly. Pieces of 3MM Whatman filter paper and nylon membrane (BDH) were cut to the size of the manifold. The filter paper and membrane were immersed in 6x SSC for 10 min and then placed onto the bottom plate of the manifold with the nylon membrane on top of the filter paper. The top plate of the manifold was placed ontop of the nylon membrane and assembled according to the manufacturers instructions to ensure it was air-tight. 200 µl of 6x SSC was added to 5 µg of DNA and the samples denatured at 100°C for 10 min then placed on ice immediately. The suction to the manifold device was applied and 500 µl of 6x SSC pipetted into each well used (unused wells were blocked with masking tape). The DNA samples were microcentrifuged and applied to the wells in the top plate without touching the nylon membrane. On completion of filtration, the apparatus was dismantled and the membrane placed on a 3MM Whatman paper presoaked in denaturation buffer for 10 min. The membrane was then transferred to a 3MM Whatman paper presoaked in neutralisation buffer for 5 min. The membrane was dried on 3MM Whatman filter paper and DNA immobilised by irradiating at 1200 Joules for 1 min in a UV transilluminator. The membrane was stored dry between sheets of 3MM Whatman filter paper at room temperature.

6.11.4 Hybridisation of DNA dot blots

The membrane carrying the immobilised DNA was presoaked in 6x SSC for 5 min then transferred to a plastic bag with hybridisation buffer and sealed (ensuring that all air bubbles were removed). The pre-hybridisation was carried out at 42°C for 2 hr. The radioactive DNA probe was then denatured at 99°C for 2 min, held on ice for 2 min and

added to the membrane by snipping a corner of the bag and introducing the probe by pipette. Any air bubbles were removed and the bag resealed. The membrane was incubated overnight (18 hr, 42°C) in a shaking water bath and then was removed from the bag and washed twice in 2x SSC for 10 min at room temperature with agitation. The blot was then rinsed in 2x SSC and washed in a solution consisting of 0.1% SDS and 0.2% SSC twice for 1 hr each at 42°C in a shaking water bath. Following these washing stages, the blot was Saran wrapped and exposed to Kodak film overnight at -70°C.

RESULTS AND DISCUSSIONS

CHAPTER 7

CHARACTERISATION OF CHOLECYSTOKININ RECEPTORS AND THEIR GROWTH RESPONSE IN ESTABLISHED HUMAN PANCREATIC CANCER CELL LINES

CHAPTER 7

7.1 Background

Pancreatic cancer is one of the most lethal cancers in the Western world with only 10-20% being resectable at diagnosis (Silverberg *et al.*, 1990) and the present alternative treatments are ineffective. This poor prognosis has stimulated research scientists to develop other approaches to combat the problem, including the possible role of hormones such as cholecystokinin in pancreatic cancer growth.

7.2 Aims

The aims of this study were i) to investigate the effect of gastrointestinal hormones, sCCK-8 and nsG-17 on the growth of established human pancreatic cancer cell lines; ii) to determine the CCK-R status in the cell lines using well developed molecular techniques and radioligand binding assays; iii) to evaluate the effects of novel specific CCK-R antagonists on the growth of the human pancreatic cancer cell lines.

7.3 Effects of sCCK-8 and nsG-17 on the growth of two human pancreatic cancer cell lines *in vitro*

7.3.1 *Methods*

The methods are described in section 2.5 for Mia PaCa-2 and BxPc-3 cells. The cells were plated out in serum and allowed to reach log phase of growth. Following this, the cells were exposed to serum-free conditions in order to reach a quiescent stage before stimulating with the peptide agonists. The cells were counted every 48 hr for a period of 10 days.

7.3.2 *Results*

7.3.2.1 *Mia PaCa-2*

Figures 7.1 and 7.2 show that there was no significant growth response to either varying concentrations of sCCK-8 or nsG-17 in the Mia PaCa-2 cells respectively. P values ranged from a minimum of 0.11 at day 2 of incubation with 10 pM and 0.72 at

day 8 of incubation with 100 nM sCCK-8. P values obtained with varying concentrations of nsG-17 ranged from a minimum of 0.08 at 10 pM on day 2 and a maximum of 1.0 at 100 nM on day 10 of incubation. Figures 7.1F and 7.2F show that the cells had reached a quiescent stage in growth in serum-free medium whereas tissue culture medium supplemented with 10% foetal calf serum maintained the increase in growth of these cells ($P<0.02$ from day 2 onwards).

7.3.2.2 *BxPc-3*

Figures 7.3 and 7.4 show that the cells did not respond to either CCK-R agonists sCCK-8 or nsG-17 respectively. P values ranged from a minimum of 0.15 at day 8 of incubation with 10 nM and 1.0 at day 2 of incubation with 10 pM sCCK-8. P values obtained with varying concentrations of nsG-17 ranged from a minimum of 0.04 at 100 pM on day 6 and a maximum of 1.0 at 100 nM on day 8 of incubation. Figures 7.3F and 7.4F show that the cells had reached quiescence in serum-free medium. 10% foetal calf serum stimulated the growth of the BxPc-3 cells, during the growth assay.

Figure 7.1 Effects of sCCK-8 on growth of Mia PaCa-2 cells in culture. Cells were grown in the absence (○), presence of various concentrations of sCCK-8 (10 pM-100 nM, A-E) in serum-free DMEM (●) or 10% foetal calf serum in DMEM (F, ●) for 10 days. Figure F is on a different scale to A-E. Each point represents the mean ± s.e. for each group. Three wells of cells were tested for each concentration and each experiment was performed six times (n=18/treatment group). sCCK-8 had no effect on growth of Mia PaCa-2 cells.

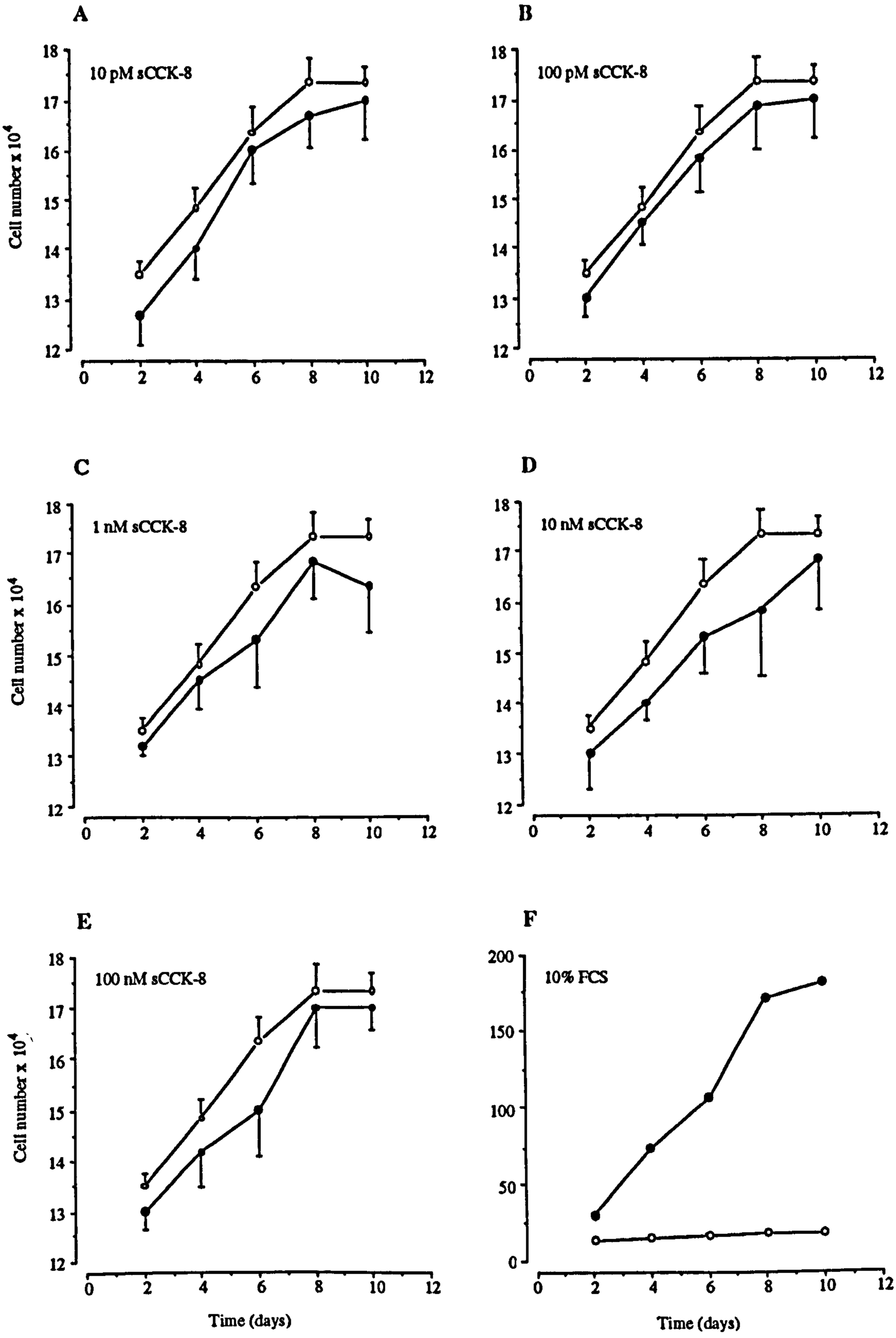

A
10 pM sCCK-8
B
100 pM sCCK-8
C
1 nM sCCK-8
D
10 nM sCCK-8
E
100 nM sCCK-8
F
10% FCS
Cell number x 10⁴
Time (days)
18
17
16
15
14
13
12
200
150
100
50
0
0
2
4
6
8
10
12

Figure 7.2 Effects of nsG-17 on growth of Mia PaCa-2 cells in culture. Cells were grown in the absence (○), presence of various concentrations of nsG-17 (10 pM-100 nM, A-E) in serum-free DMEM (●) or 10% foetal calf serum in DMEM (F, ●) for 10 days. Figure F is on a different scale to A-E. Each point represents the mean ± s.e. for each group. Three wells of cells were tested for each concentration and each experiment was performed six times (n=18/treatment group). nsG-17 had no effect on growth of Mia PaCa-2 cells.

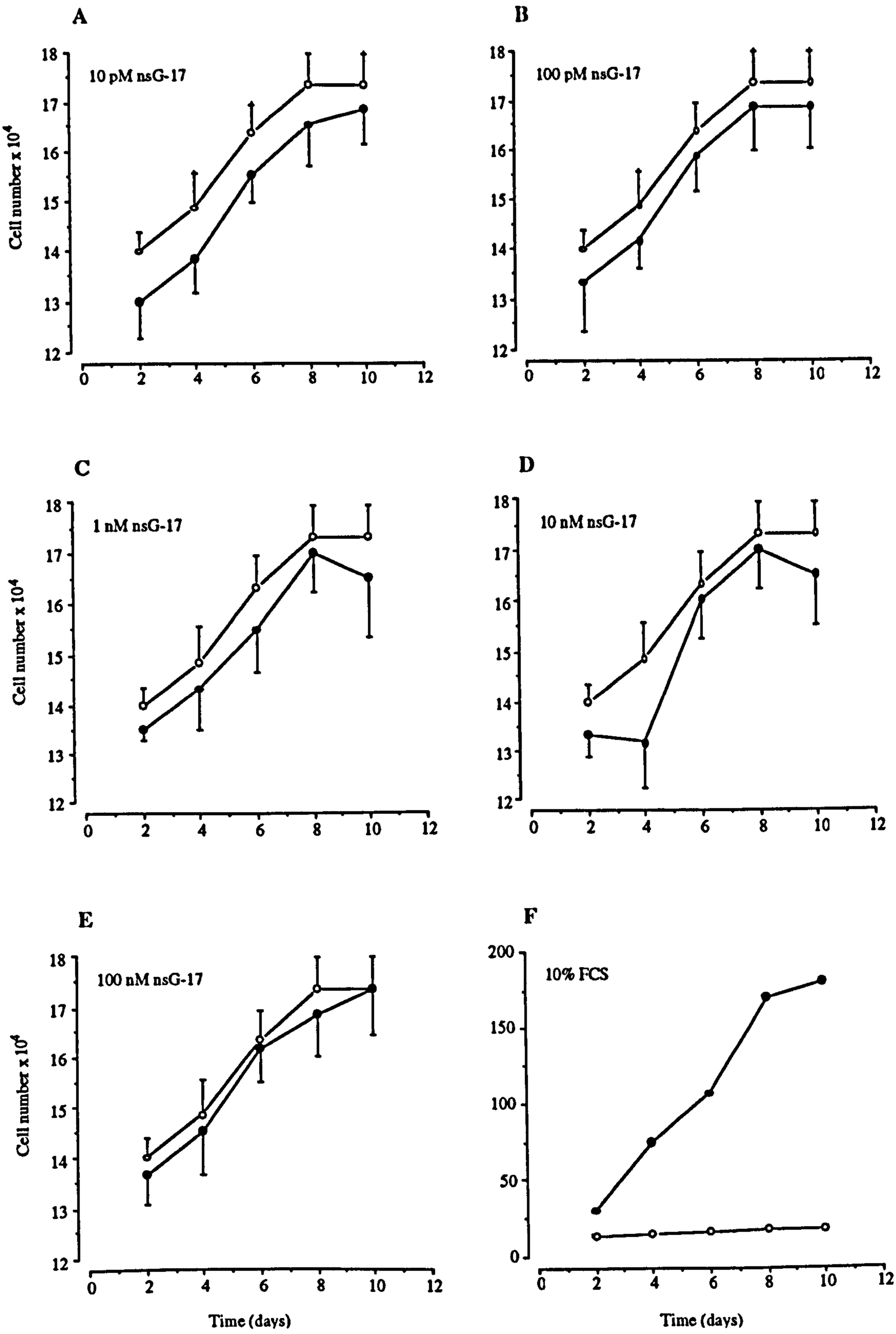

A
10 pM nsG-17
B
100 pM nsG-17
C
1 nM nsG-17
D
10 nM nsG-17
E
100 nM nsG-17
F
10% FCS
Cell number x 10⁴
Time (days)

Figure 7.3 Effects of sCCK-8 on growth of BxPc-3 cells in culture. Cells were grown in the absence (○), presence of various concentrations of sCCK-8 (10 pM-100 nM, A-E) in serum-free RPMI (●) or 10% foetal calf serum in RPMI (F, ●) for 10 days. Figure F is on a different scale to A-E. Each point represents the mean ± s.e. for each group. Three wells of cells were tested for each concentration and each experiment was performed six times (n=18/treatment group). sCCK-8 had no effect on growth of BxPc-3 cells.

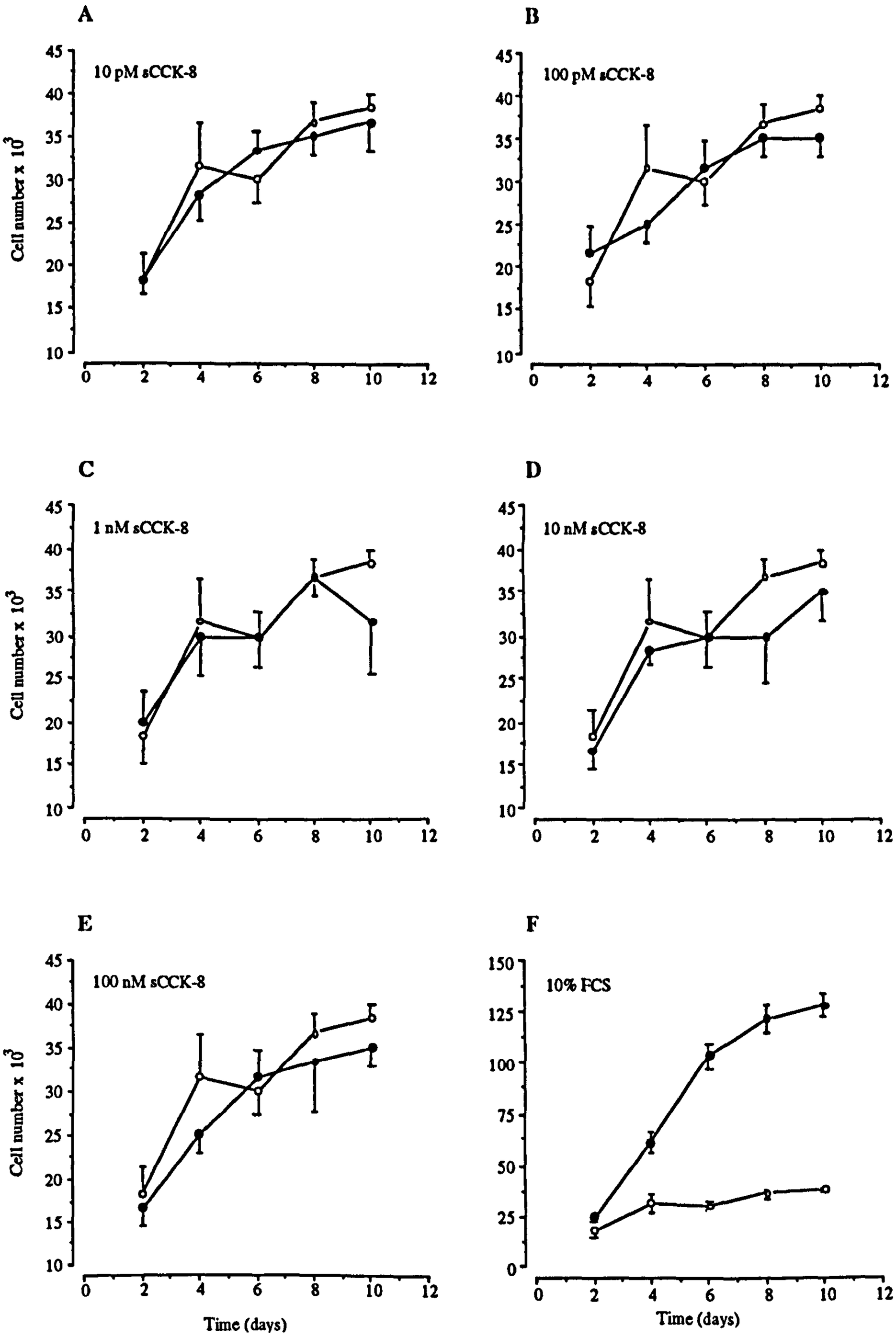
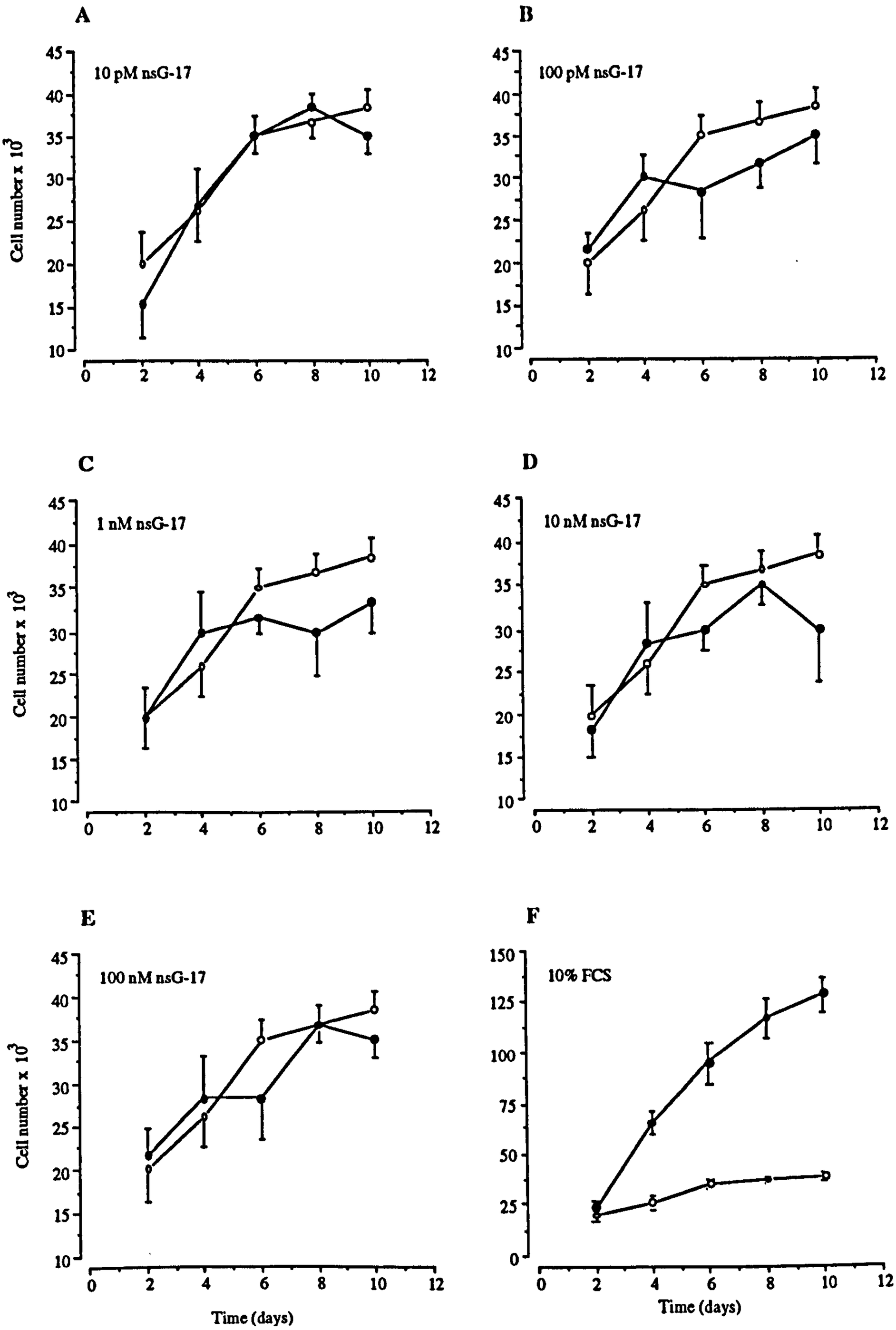

A
10 pM sCCK-8
B
100 pM sCCK-8
C
1 nM sCCK-8
D
10 nM sCCK-8
E
100 nM sCCK-8
F
10% FCS
Cell number x 10³
Time (days)
0
2
4
6
8
10
12
10
15
20
25
30
35
40
45
50
75
100
125
150

Figure 7.4 Effects of nsG-17 on growth of BxPc-3 cells in culture. Cells were grown in the absence (○), presence of various concentrations of nsG-17 (10 pM-100 nM) in serum-free RPMI (●) or 10% foetal calf serum in RPMI (F, ●) for 10 days. Figure F is on a different scale to A-E. Each point represents the mean ± s.e. for each group. Three wells of cells were tested for each concentration and each experiment was performed six times (n=18/treatment group). nsG-17 had no effect on growth of BxPc-3 cells.


A
10 pM nsG-17
B
100 pM nsG-17
C
1 nM nsG-17
D
10 nM nsG-17
E
100 nM nsG-17
F
10% FCS
Cell number x 10³
Time (days)

7.4 Effect of sCCK-8 and nsG-17 on the growth of NIH3T3CCK-BR cells *in vitro*

7.4.1 *Methods*

The methods are described in section 2.7. The cells were plated out in serum-free medium in the presence of the agonists and counted every 48 hr for 10 days.

7.4.2 *Results*

7.4.2.1 *NIH3T3CCK-BR cells*

The NIH3T3CCK-BR cells were kindly donated by Dr. Matsui in Japan. These cells are mouse fibroblasts transfected with human CCK-BR. Figures 7.5 and 7.6 show that both sCCK-8 and nsG-17 significantly promoted the growth of NIH3T3CCK-BR cells in serum-free medium, respectively. sCCK-8 significantly induced growth responses at 1, 10 and 100 nM concentrations. P values varied from a minimum of <0.005 to a maximum value of 0.05. nsG-17 induced significant growth responses at concentrations of 1, 10 and 100 nM. Significant P values varied from a minimum of <0.005 to a maximum value of 0.013. The EC_{50} values for sCCK-8 and nsG-17 were calculated as 6.0 ± 1.3 nM and 5.1 ± 1.6 nM (n=18), respectively. Figure 7.5F shows that the cells had reached quiescence in serum-free medium and that the cell growth was maintained in 10% bovine calf serum during the assay ($P < 0.005$).

Figure 7.5 Effects of sCCK-8 on growth of NIH3T3CCK-BR cells in culture. Cells were grown in the absence (○), presence of various concentrations of sCCK-8 (10 pM-100 nM, A-E) in serum-free DMEM (●) or 10% bovine calf serum in DMEM (F, ●) for 10 days. Figure F is on a different scale to A-E. Each point represents the mean ± s.e. for each group. Three wells of cells were tested for each concentration and each experiment was performed six times (n=18/treatment group). sCCK-8 stimulated the growth of NIH3T3CCK-BR cells.

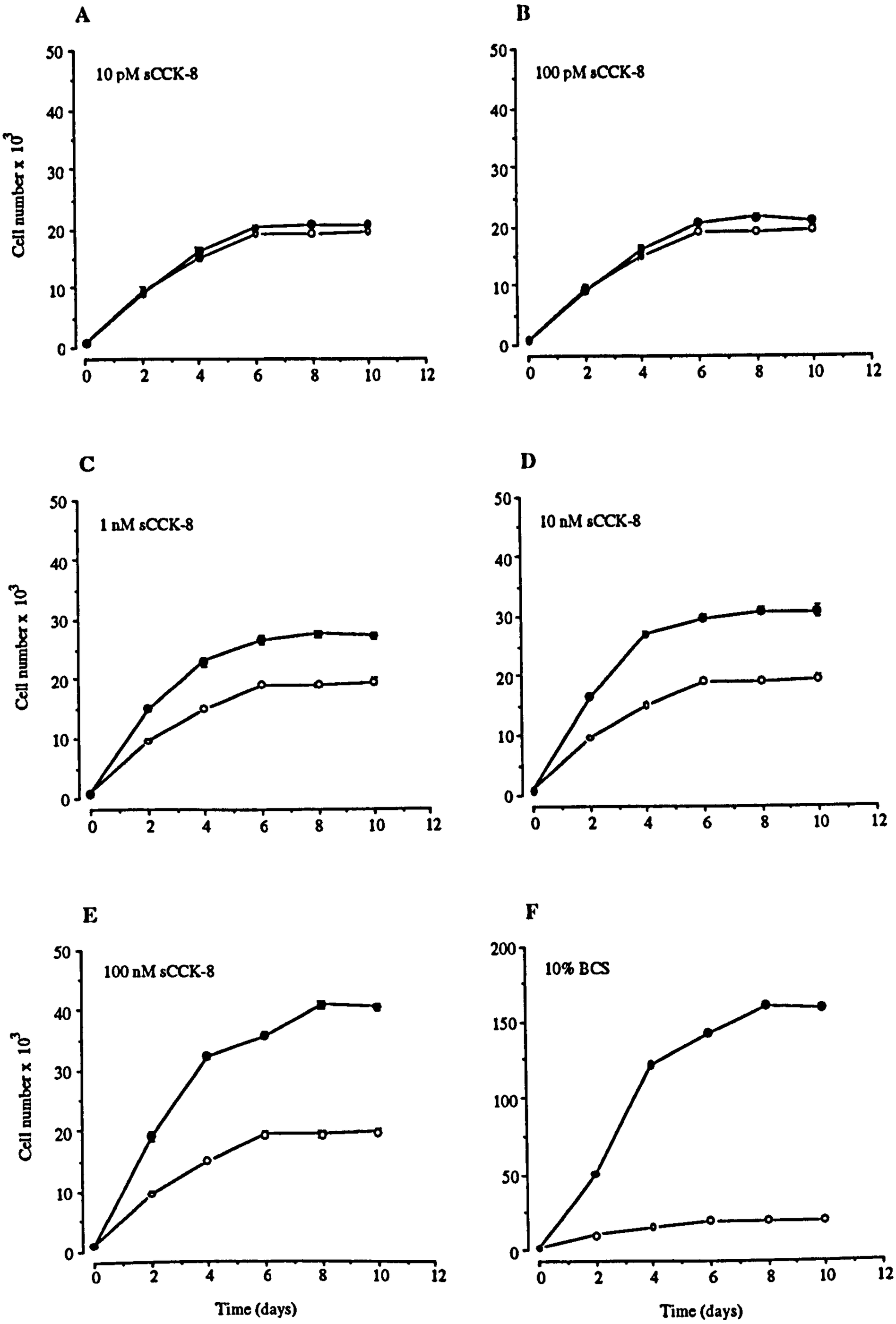
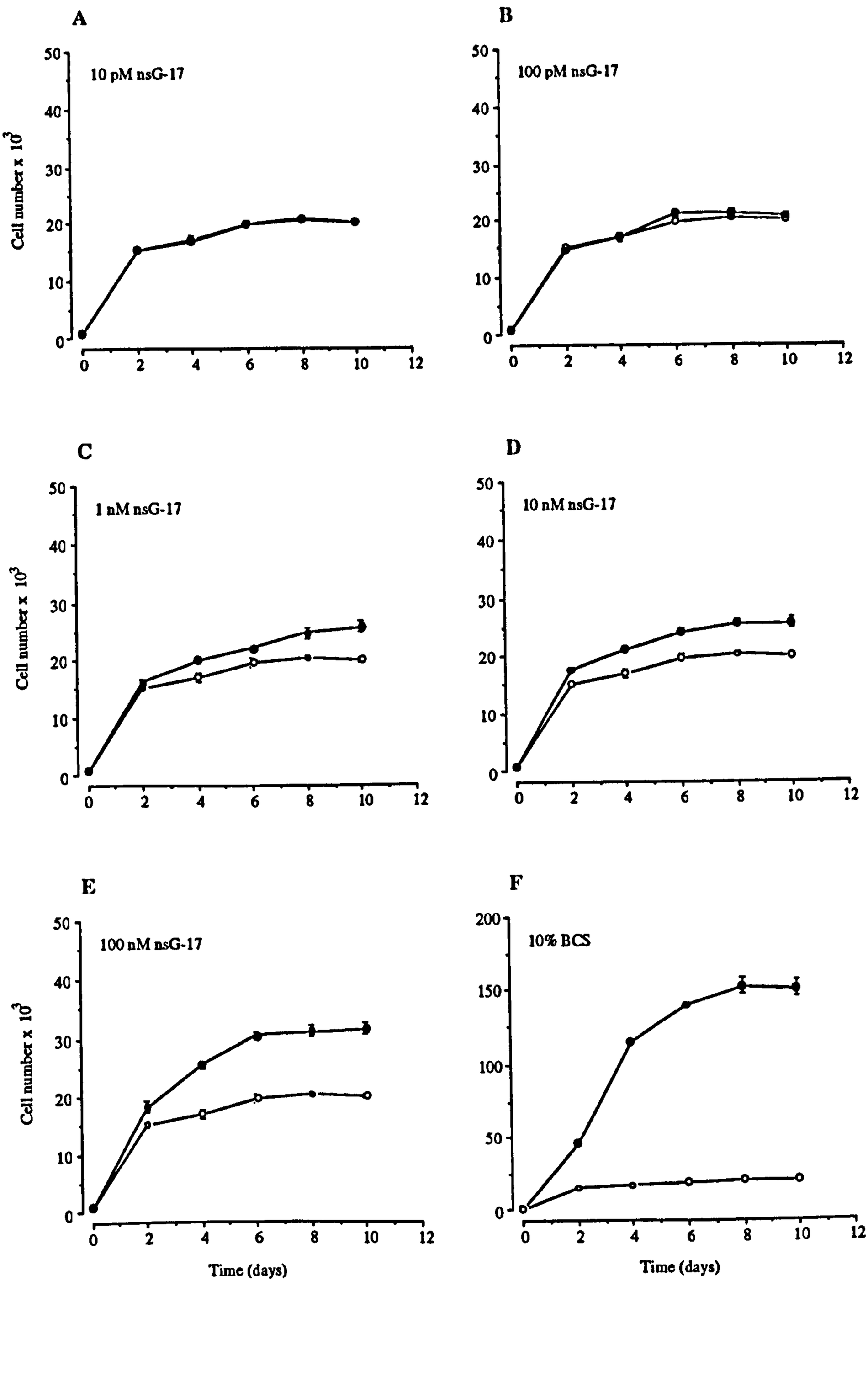

A
10 pM sCCK-8
B
100 pM sCCK-8
C
1 nM sCCK-8
D
10 nM sCCK-8
E
100 nM sCCK-8
F
10% BCS
Cell number x 10^3
Time (days)
0
2
4
6
8
10
12
10
20
30
40
50
100
150
200

Figure 7.6 Effects of nsG-17 on growth of NIH3T3CCK-BR cells in culture. Cells were grown in the absence (○), presence of various concentrations of nsG-17 (10 pM-100 nM, A-E) in serum-free DMEM (●) or 10% bovine calf serum in DMEM (F, ●) for 10 days. Figure F is on a different scale to A-E. Each point represents the mean ± s.e. for each group. Three wells of cells were tested for each concentration and each experiment was performed six times (n=18/treatment group). nsG-17 stimulated the growth of NIH3T3CCK-BR cells.


A
10 pM nsG-17
B
100 pM nsG-17
C
1 nM nsG-17
D
10 nM nsG-17
E
100 nM nsG-17
F
10% BCS
Cell number x 10³
Time (days)

7.5 Effects of CCK-R antagonists on the growth of two human pancreatic cancer cell lines *in vitro*

7.5.1 *Methods*

The methods are described in section 2.6 for Mia PaCa-2 and BxPc-3 cells. The cells were plated out in serum and allowed to reach log phase of growth. Following this the cells were either grown in 10% foetal calf serum or in serum-free conditions in order to reach a quiescent stage before incubating with the various CCK-R specific antagonists. The cells were counted on day 6.

7.5.2 *Results*

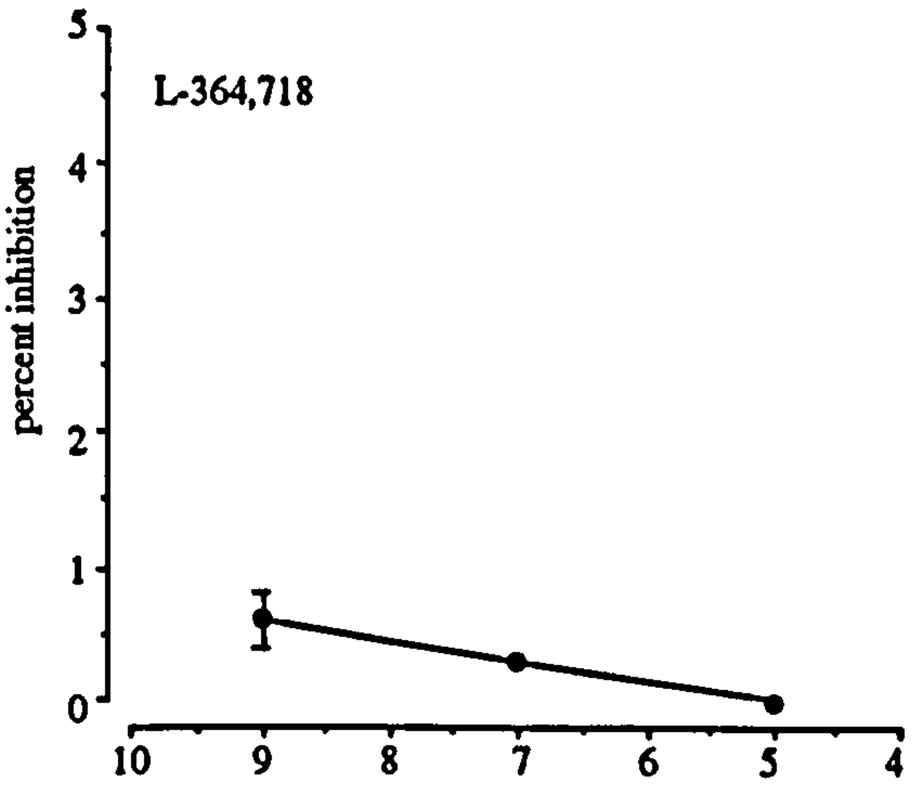
7.5.2.1 *Mia PaCa-2*

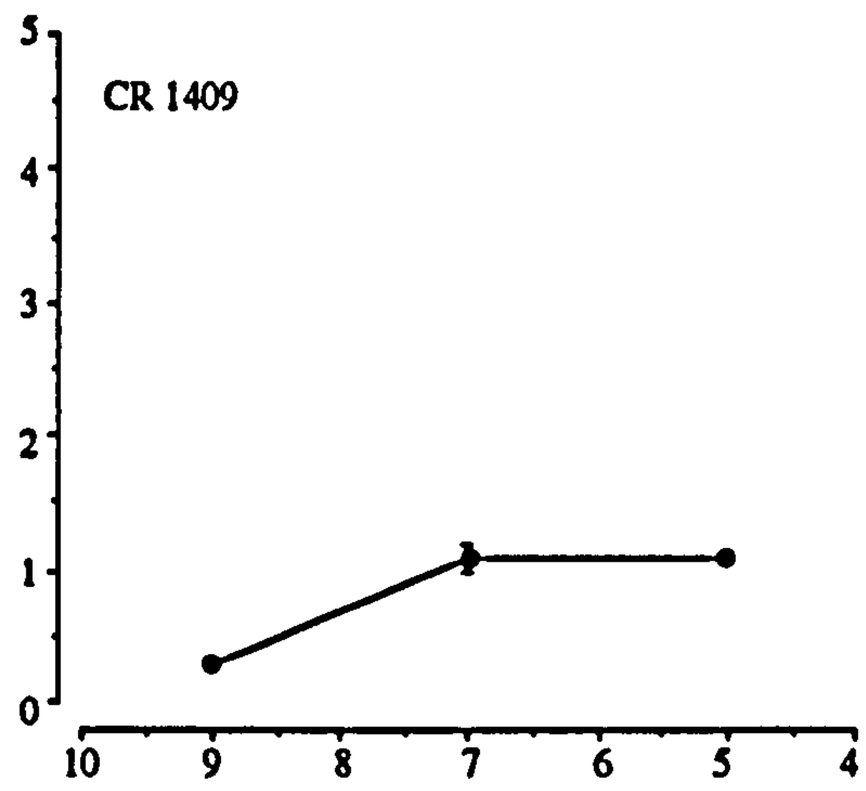
Figures 7.7 show that the various CCK-R antagonists had no significant effect on the serum maintained growth of Mia PaCa-2 ($P > 0.22$). The antagonists also had no effect on the cells in culture in serum-free medium as shown in Figures 7.8 ($P > 0.12$).

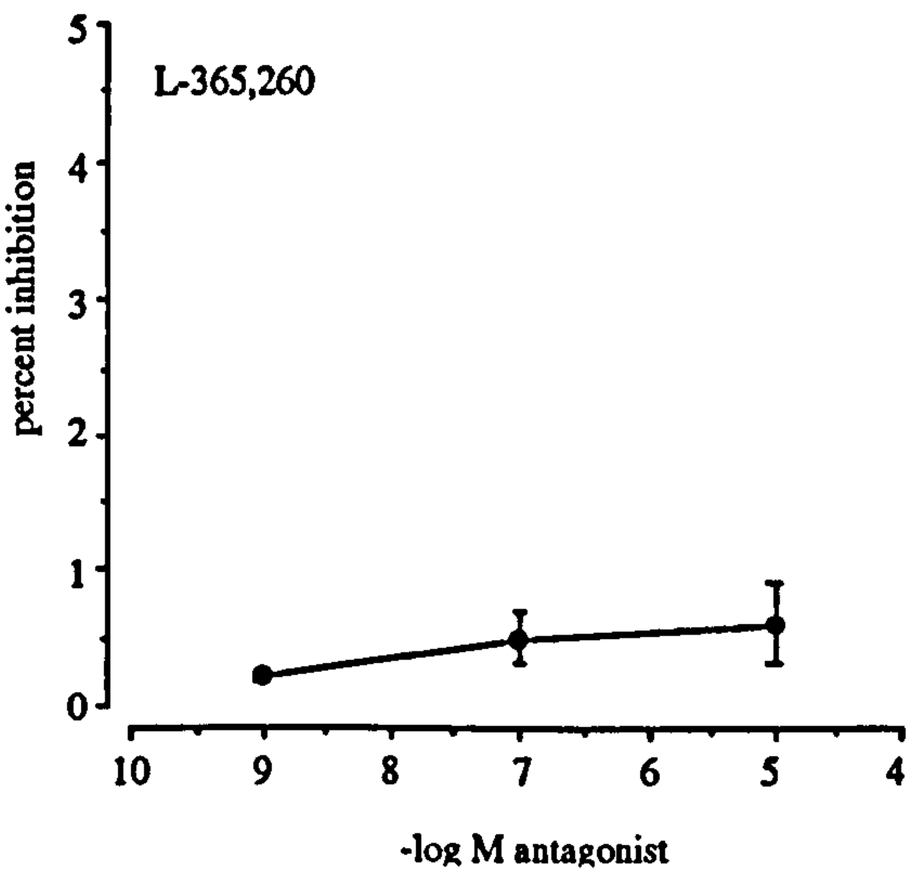
7.5.2.2 *BxPc-3*

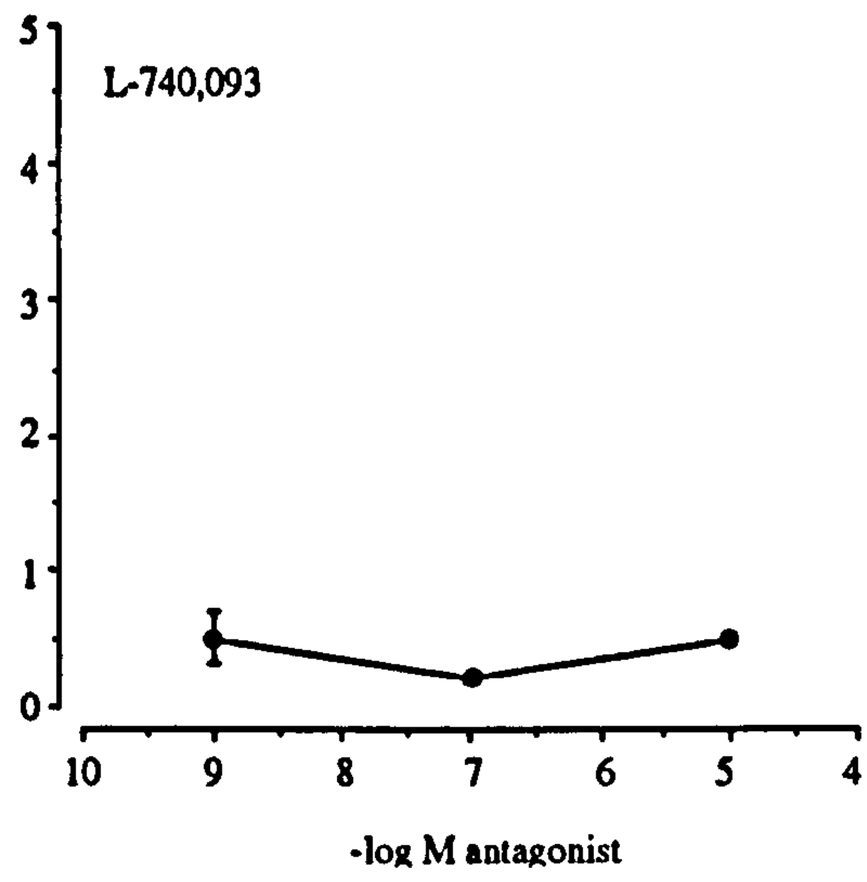
Figures 7.9 show that the various CCK-R antagonists had no significant effect on the serum maintained growth of BxPc-3 cells ($P > 0.40$). The antagonists also had no effect on the cells in culture in serum-free medium as shown in Figures 7.10 ($P > 0.35$).

Figure 7.7 Effects of CCK-R antagonists on growth of Mia PaCa-2 cells in culture. Cells were grown in the presence of various concentrations of antagonists (1 nM-10 μM) in DMEM containing 10% foetal calf serum for 6 days when they were counted. Each point represents the mean ± s.e. for each group. Two wells of cells were tested for each concentration and each experiment was performed six times (n=12/treatment group). CCK-R antagonists had no effect on the growth of Mia PaCa-2 cells in foetal calf serum.


L-364,718
percent inhibition
5
4
3
2
1
0
10
9
8
7
6
5
4


CR 1409
5
4
3
2
1
0
10
9
8
7
6
5
4


L-365,260
percent inhibition
5
4
3
2
1
0
10
9
8
7
6
5
4
-log M antagonist


L-740,093
5
4
3
2
1
0
10
9
8
7
6
5
4
-log M antagonist

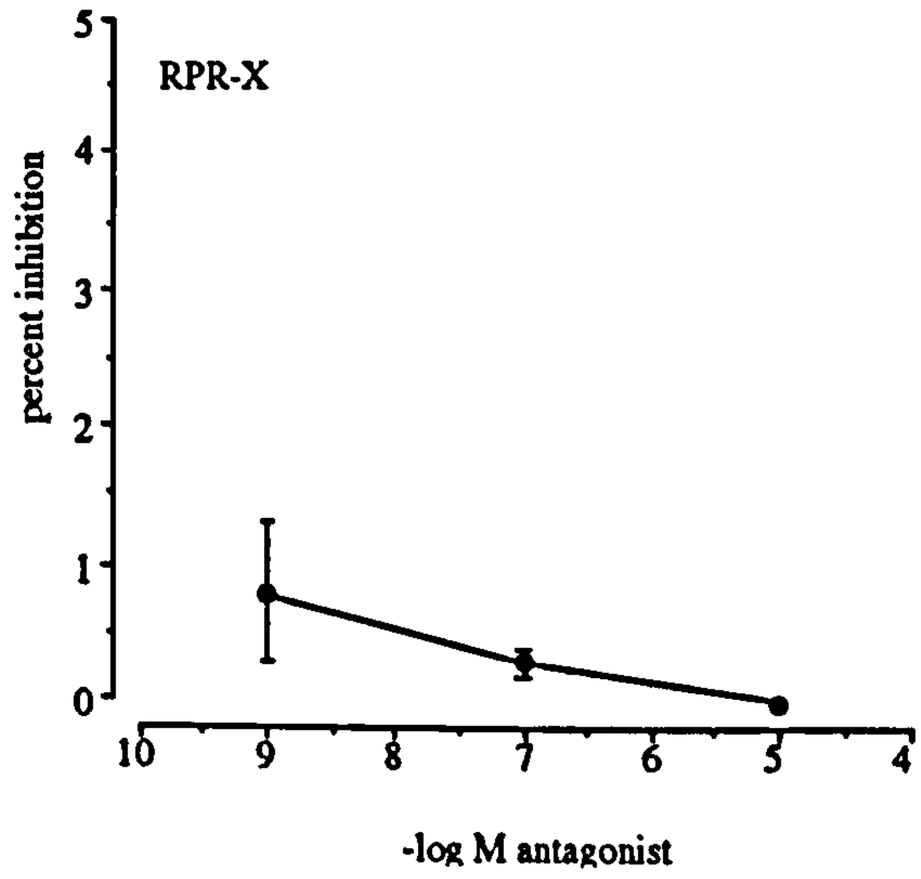
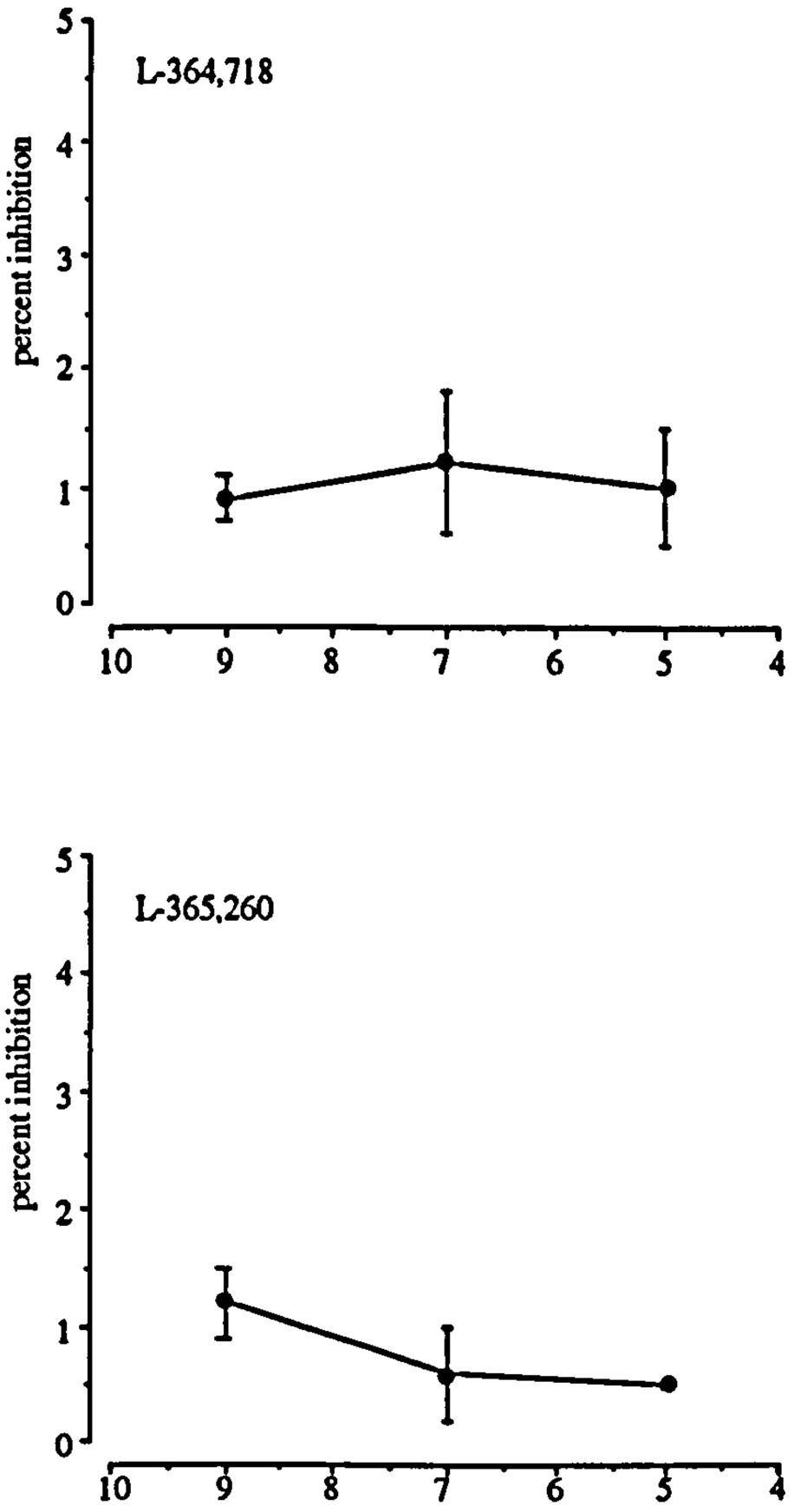
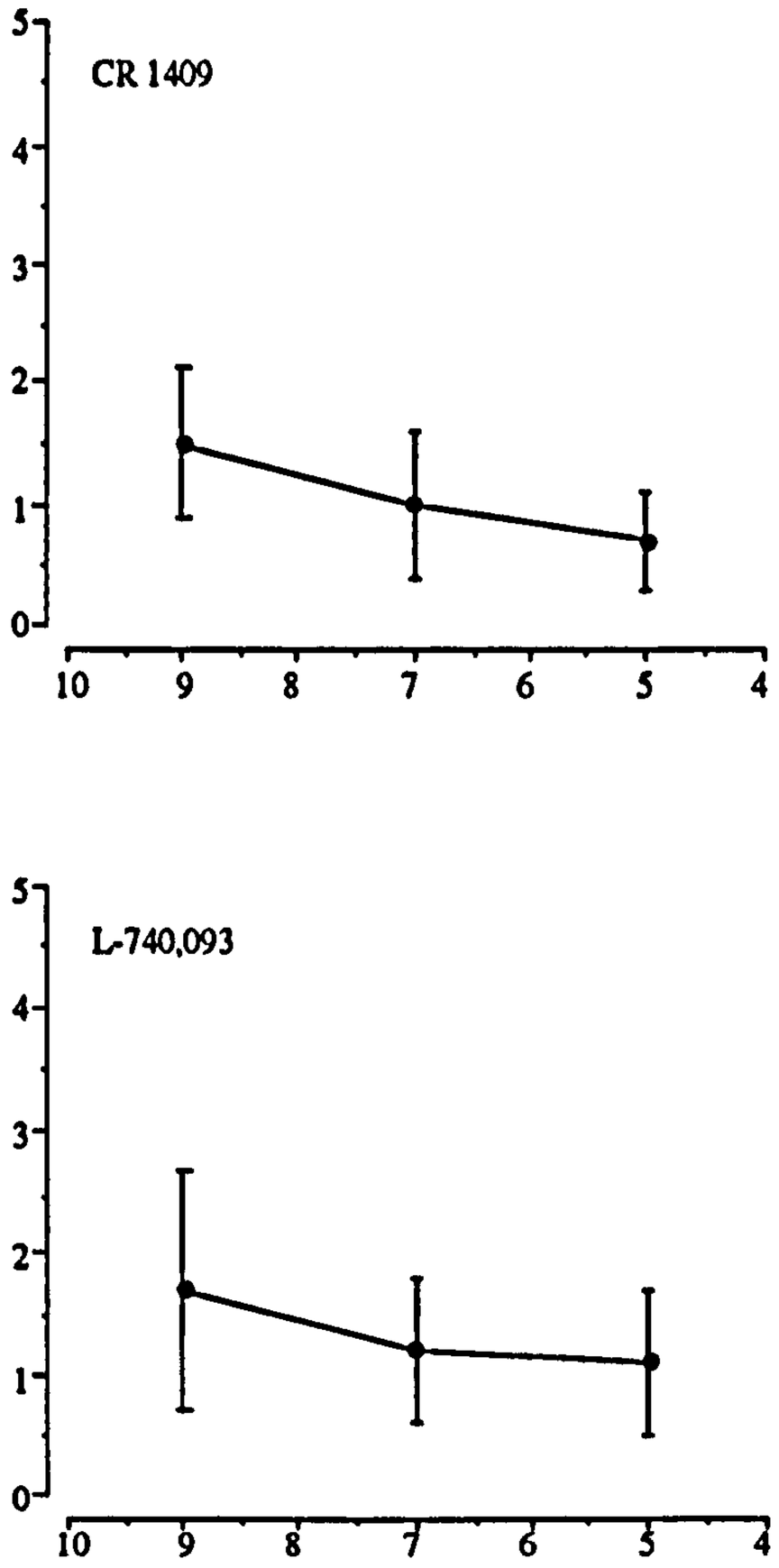

RPR-X
percent inhibition
5
4
3
2
1
0
10
9
8
7
6
5
4
-log M antagonist

Figure 7.8 Effects of CCK-R antagonists on growth of Mia PaCa-2 cells in culture. Cells were grown in the presence of various concentrations of antagonists (1 nM-10 μM) in serum-free DMEM for 6 days when they were counted. Each point represents the mean ± s.e. for each group. Two wells of cells were tested for each concentration and each experiment was performed six times (n=12/treatment group). CCK-R antagonists had no effect on the growth of Mia PaCa-2 cells in serum-free medium.


L-364,718
percent inhibition
5
4
3
2
1
0
10
9
8
7
6
5
4
-log M antagonist


CR 1409
5
4
3
2
1
0
10
9
8
7
6
5
4
-log M antagonist

L-365,260
percent inhibition
5
4
3
2
1
0
10
9
8
7
6
5
4
-log M antagonist

L-740,093
5
4
3
2
1
0
10
9
8
7
6
5
4
-log M antagonist

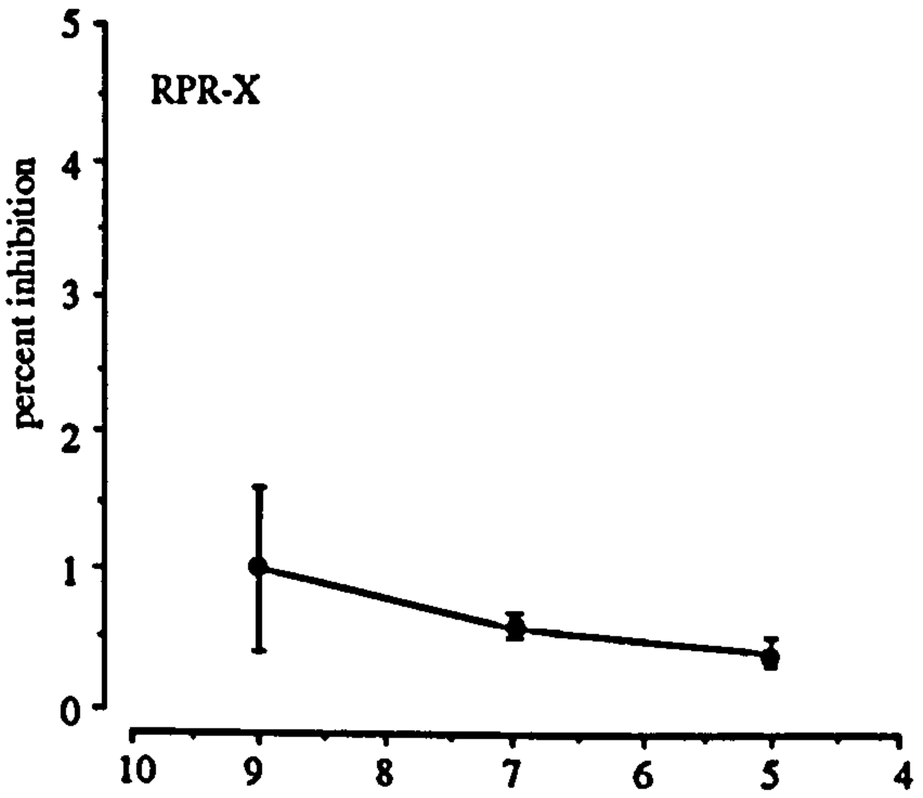
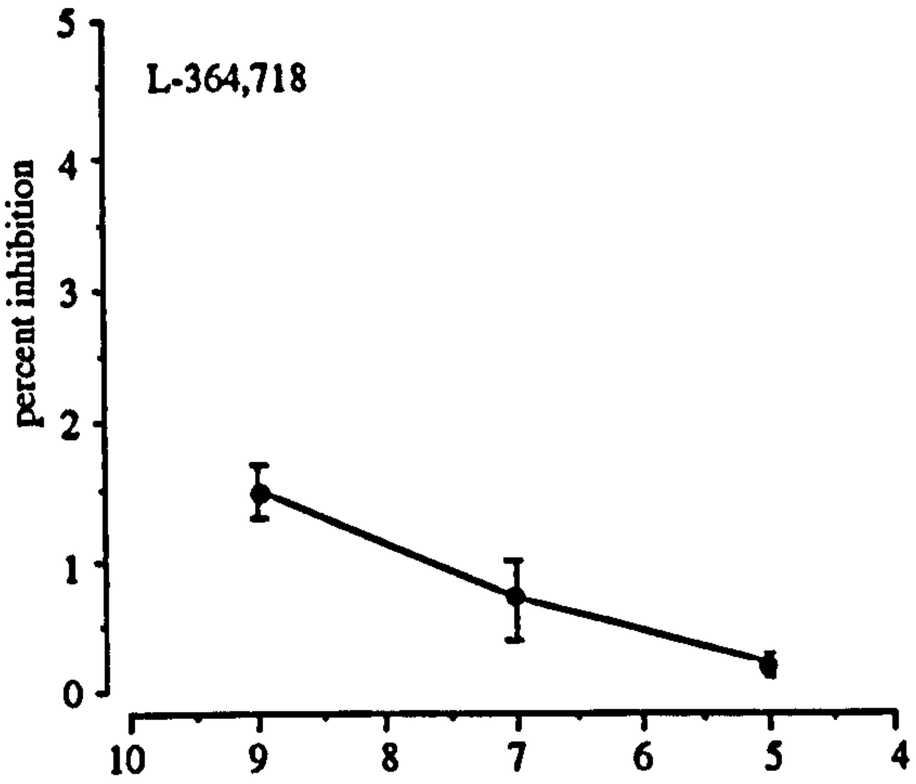
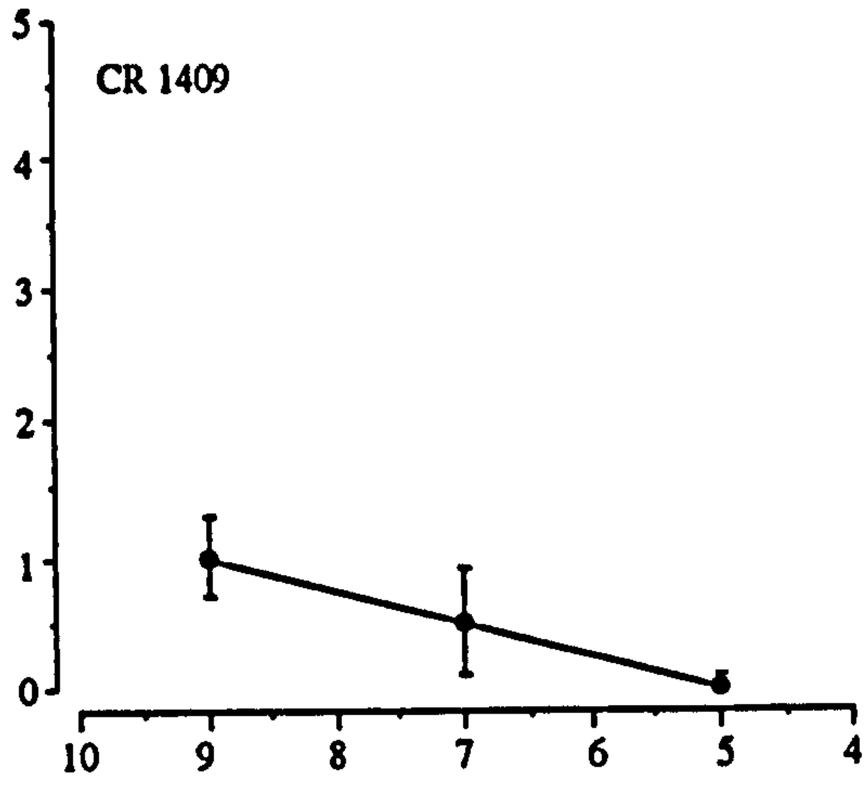
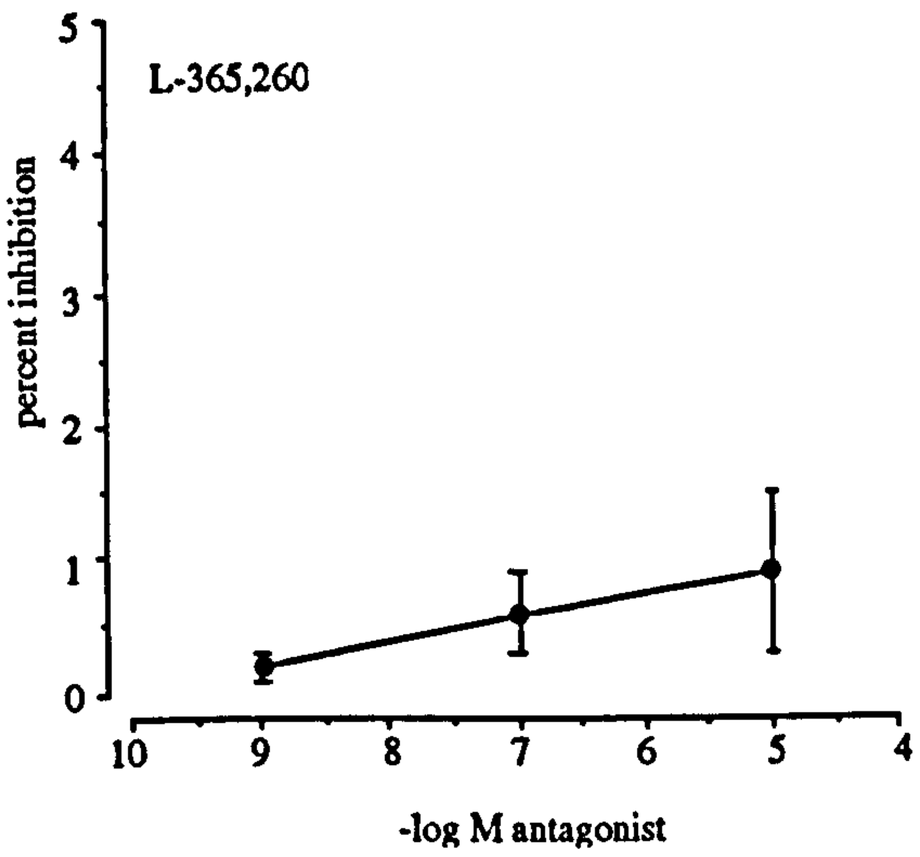
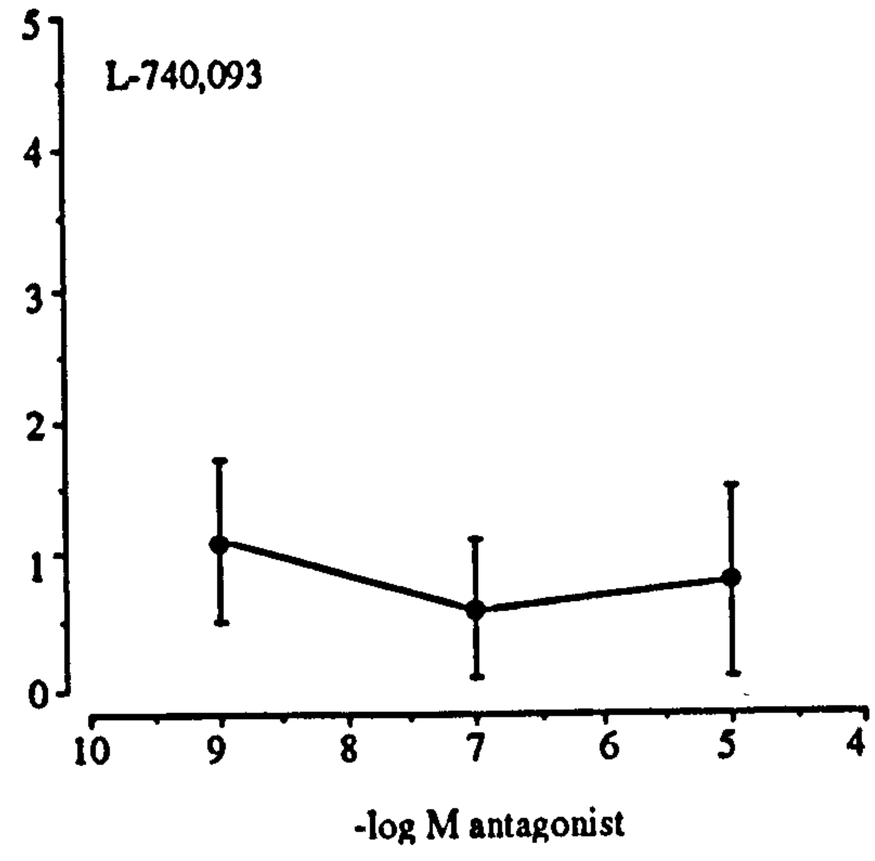

RPR-X
percent inhibition
5
4
3
2
1
0
10
9
8
7
6
5
4
-log M antagonist

Figure 7.9 Effects of CCK-R antagonists on growth of BxPc-3 cells in culture. Cells were grown in the presence of various concentrations of antagonists (1 nM-10 μM) in RPMI containing 10% foetal calf serum for 6 days when they were counted. Each point represents the mean ± s.e. for each group. Two wells of cells were tested for each concentration and each experiment was performed six times (n=12/treatment group). CCK-R antagonists had no effect on the growth of BxPc-3 cells in foetal calf serum.


L-364,718
percent inhibition
5
4
3
2
1
0
10
9
8
7
6
5
4


CR 1409
5
4
3
2
1
0
10
9
8
7
6
5
4


L-365,260
percent inhibition
5
4
3
2
1
0
10
9
8
7
6
5
4
-log M antagonist


L-740,093
5
4
3
2
1
0
10
9
8
7
6
5
4
-log M antagonist

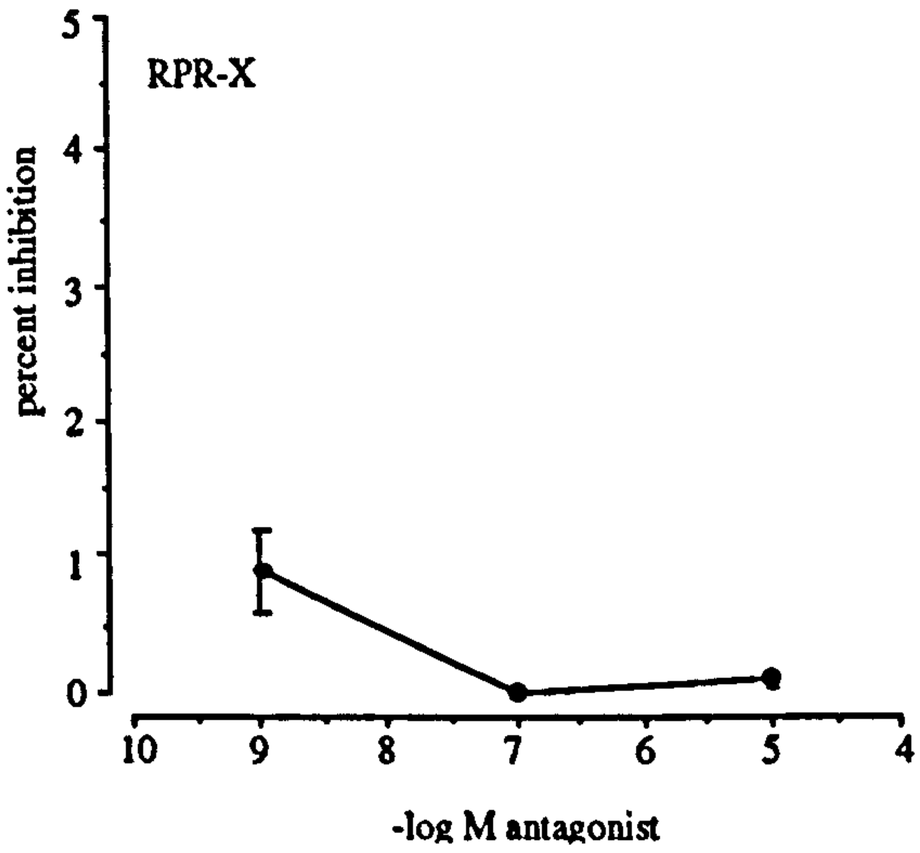
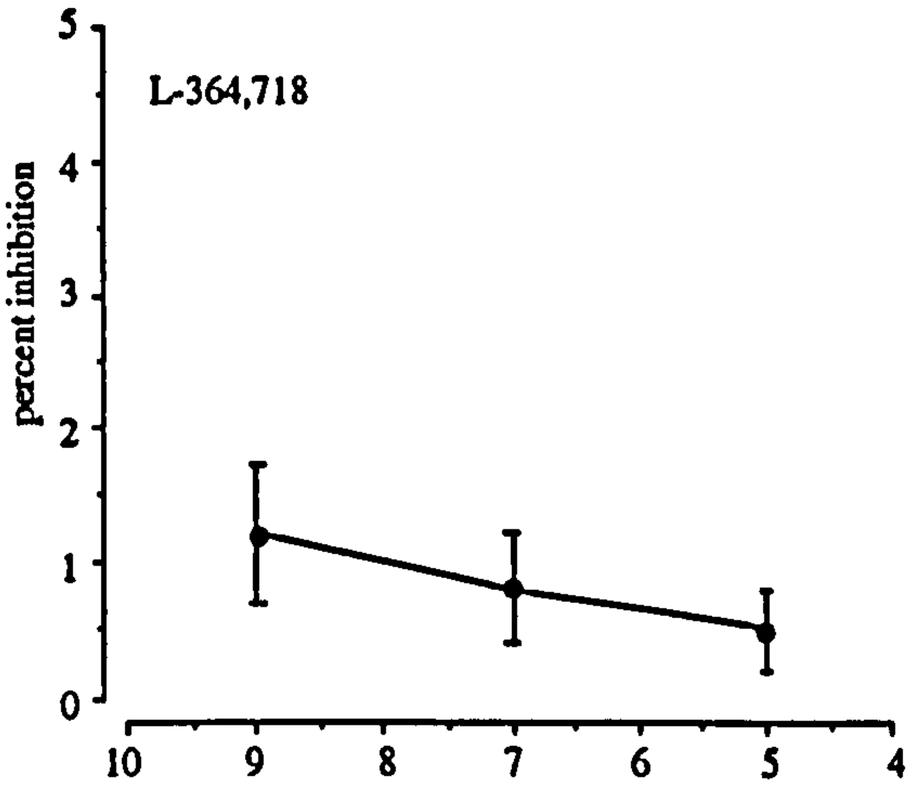
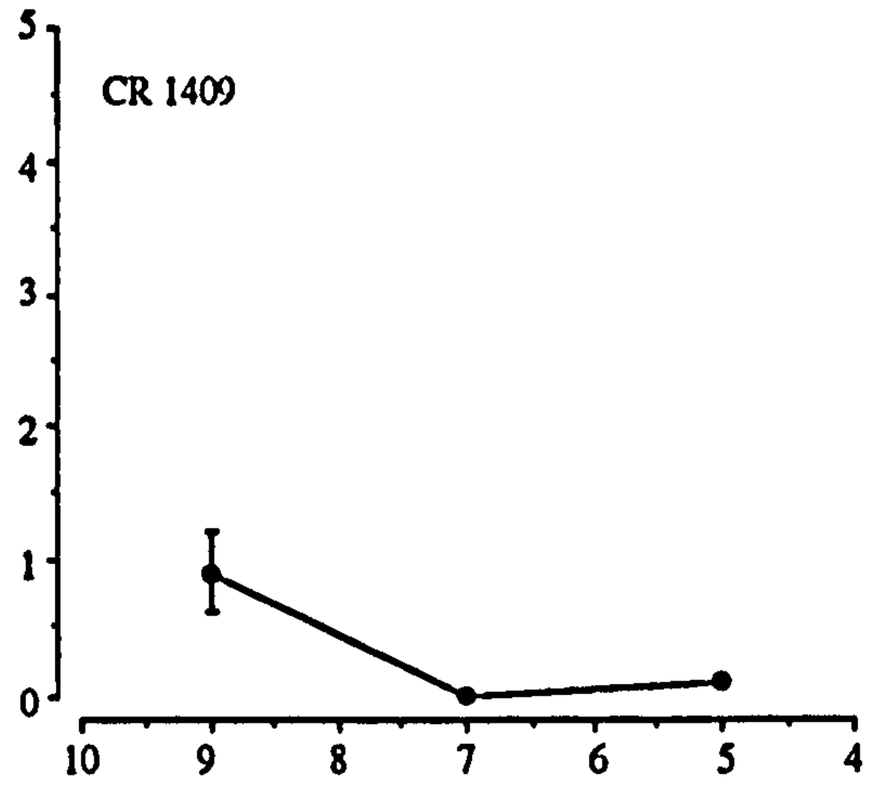
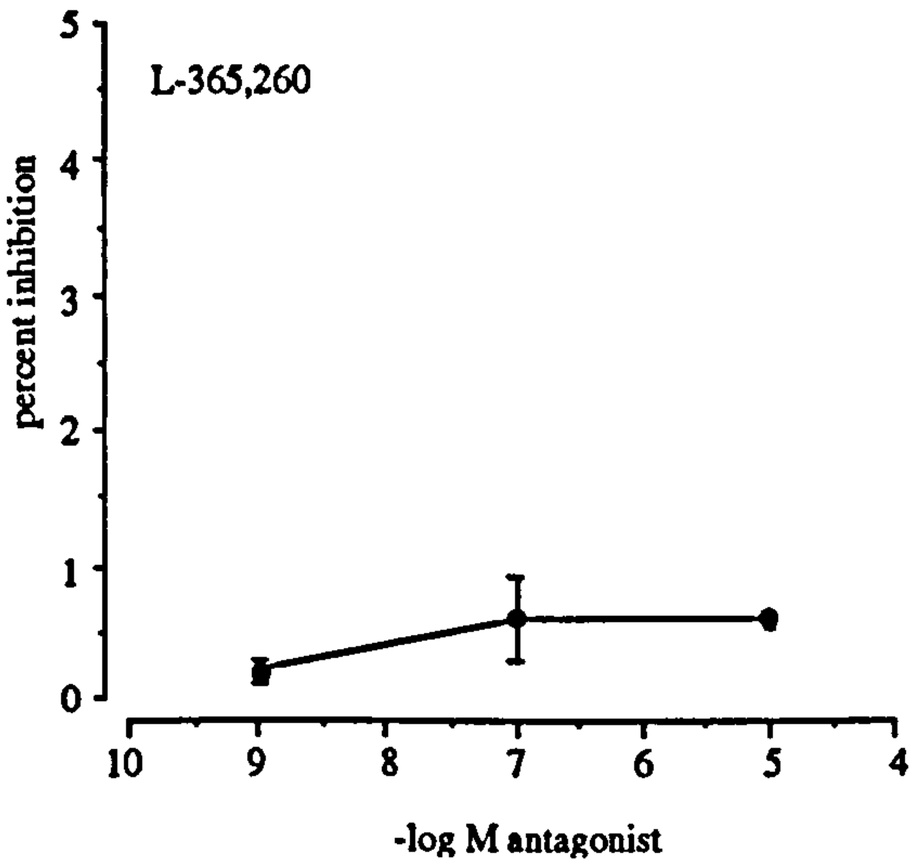
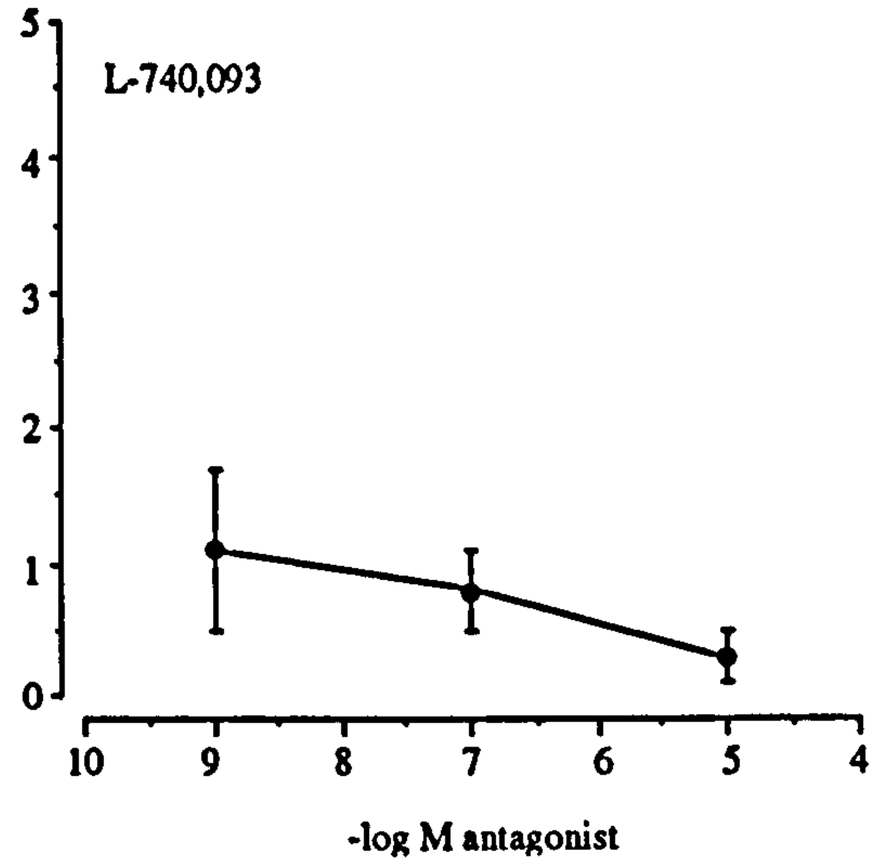

RPR-X
percent inhibition
5
4
3
2
1
0
10
9
8
7
6
5
4
-log M antagonist

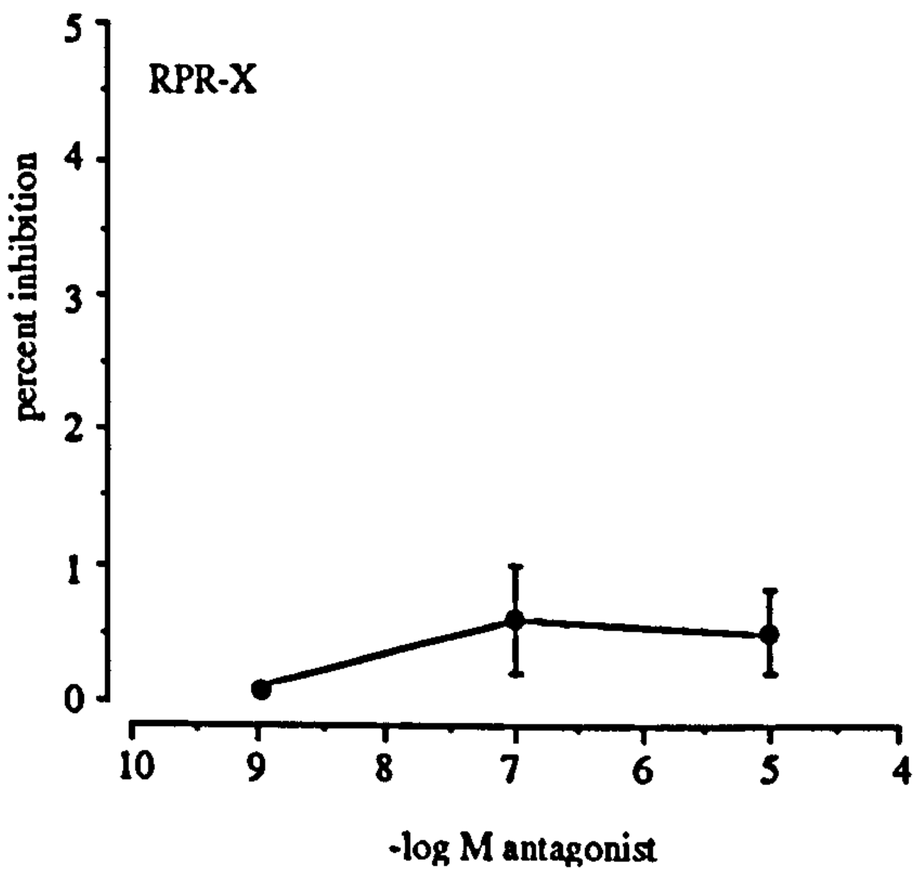
Figure 7.10 Effects of CCK-R antagonists on growth of BxPc-3 cells in culture. Cells were grown in the presence of various concentrations of antagonists (1 nM-10 μM) in serum-free RPMI for 6 days when they were counted. Each point represents the mean ± s.e. for each group. Two wells of cells were tested for each concentration and each experiment was performed six times (n=12/treatment group). CCK-R antagonists had no effect on the growth of BxPc-3 cells in serum-free medium.


L-364,718
percent inhibition
5
4
3
2
1
0
10
9
8
7
6
5
4


CR 1409
5
4
3
2
1
0
10
9
8
7
6
5
4


L-365,260
percent inhibition
5
4
3
2
1
0
10
9
8
7
6
5
4
-log M antagonist


L-740,093
5
4
3
2
1
0
10
9
8
7
6
5
4
-log M antagonist


RPR-X
percent inhibition
5
4
3
2
1
0
10
9
8
7
6
5
4
-log M antagonist

7.6 Effects CCK-AR and CCK-BR antagonists on the sCCK-8 induced growth response in NIH3T3CCK-BR cells.

7.6.1 *Methods*

The methods are described in section 2.8. The cells were incubated with 100 nM sCCK-8 in the presence or absence of the various CCK-R specific antagonists and counted on day 8.

7.6.2 *Results*

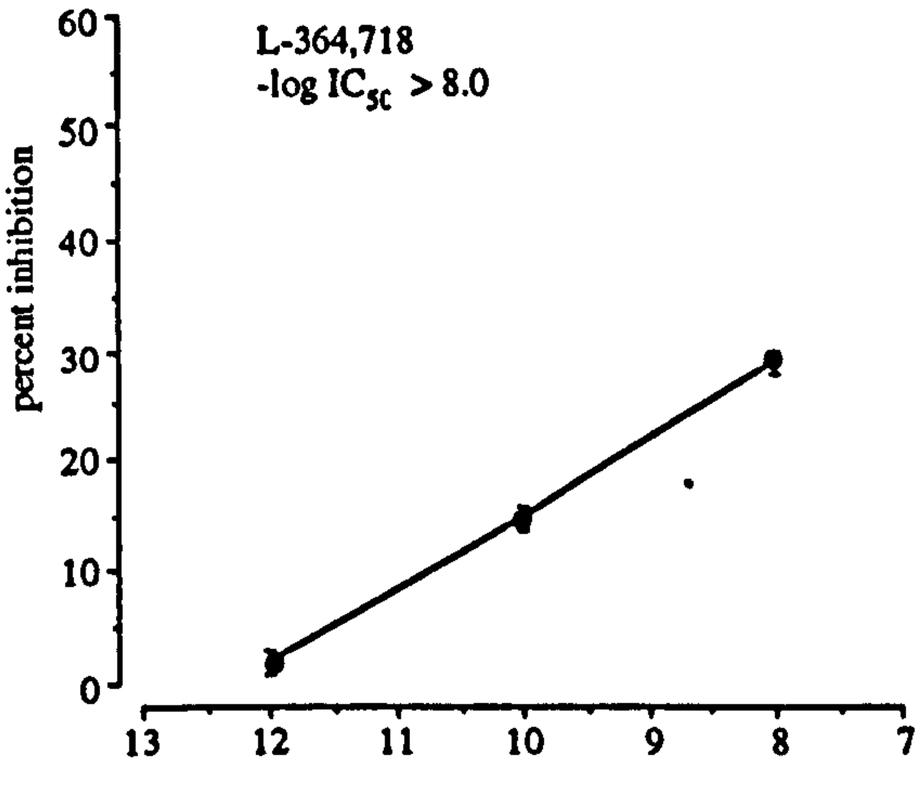
Figure 7.11 shows the inhibitory effects of the CCK-R antagonists on the sCCK-8 induced growth response in the NIH3T3CCK-BR cells. The most potent antagonist was shown to be RPR-X followed by L-740,093 > CI 988 > L-365,260 as shown in Table 7.1. These 4 CCK-BR antagonist produced significant inhibition at all three concentrations (1 pM, 100 pM and 10 nM) used ($P<0.005$). The CCK-AR antagonists, L-364,718 and CR 1409 only produced a significant inhibition of the sCCK-8 induced growth response at the higher concentrations of 100 pM and 10 nM ($P<0.005$).

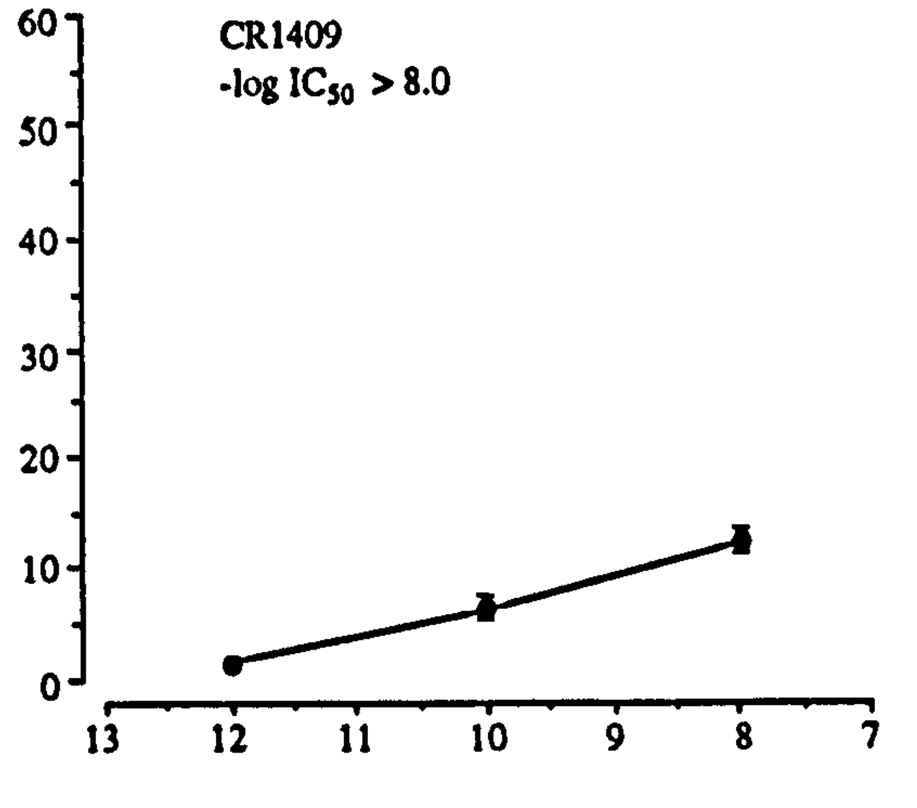
Antagonist	CCK-R Type	IC_{50} (nM)	sem	n
L-364,718	CCK-A	>10	-	12
CR 1409	CCK-A	>10	-	12
L-365,260	CCK-B	5.7	1	11
CI988	CCK-B	4	0.8	11
L-740,093	CCK-B	3.3	0.8	12
RPR-X	CCK-B	0.03	0.008	12

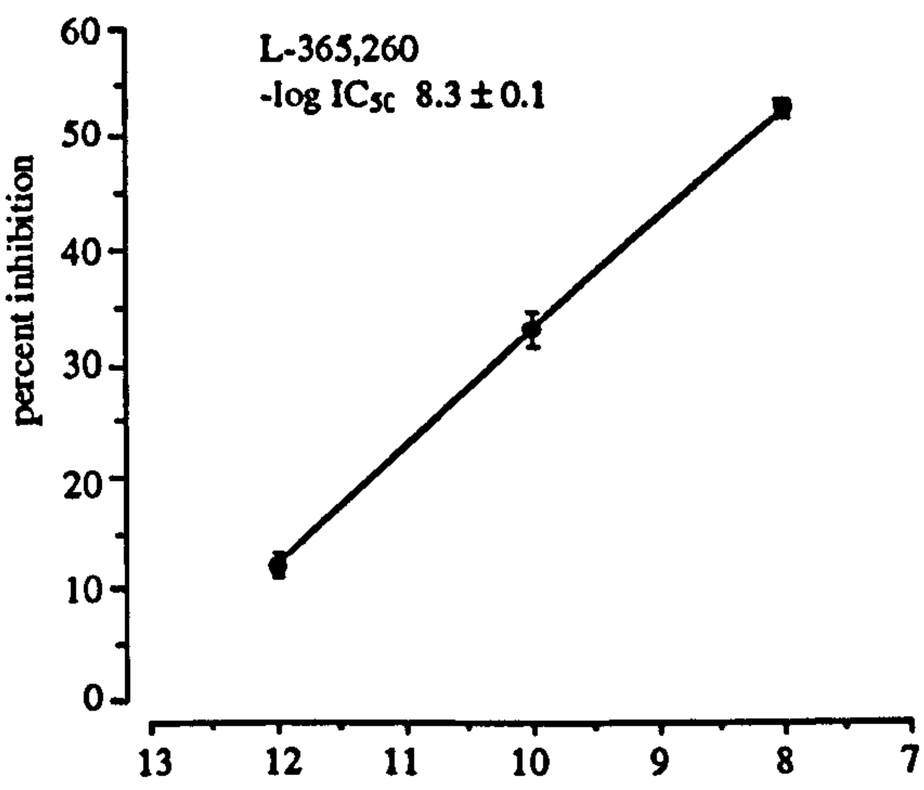
Table 7.1 Comparison of the affinities of the CCK-R antagonists for human CCK-BR in NIH3T3CCK-BR cells. RPR-X (IC_{50} 0.03 nM) was the most potent antagonist of sCCK-8 stimulated growth of NIH3T3CCK-BR cells.

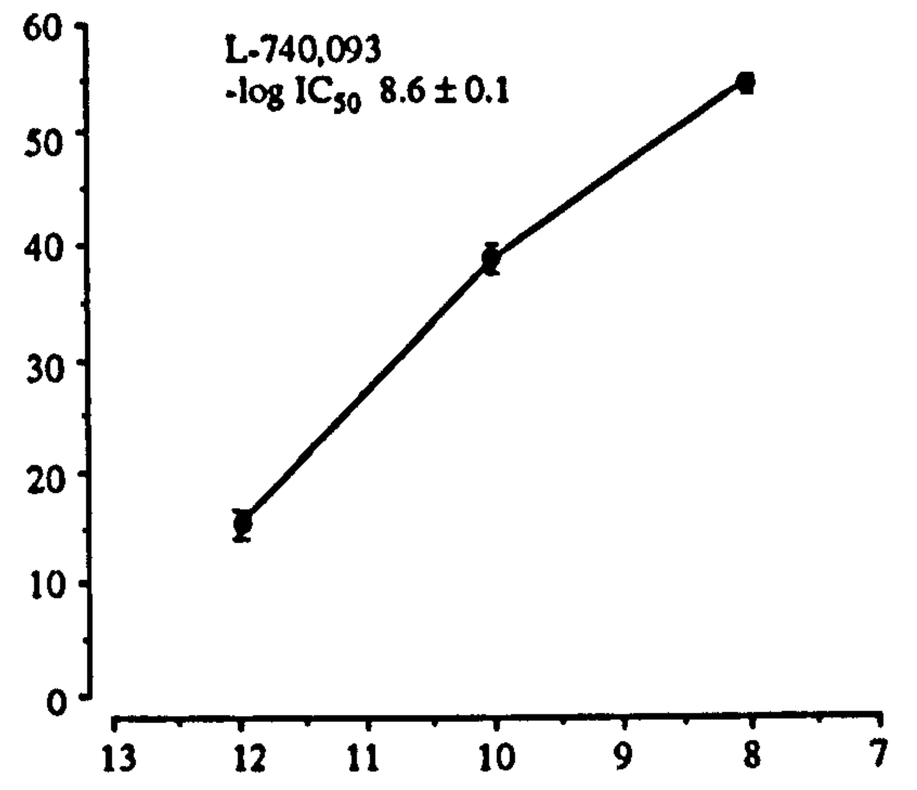
The degree of inhibition by the CCK-AR antagonists was much lower than the specific CCK-BR antagonists, resulting in IC_{50} values >10 nM. 10 nM of L-364,718 and CR 1409 inhibited the sCCK-8 response by a maximum of 28.8 ± 1.2% and 12.4 ± 1.0% respectively.

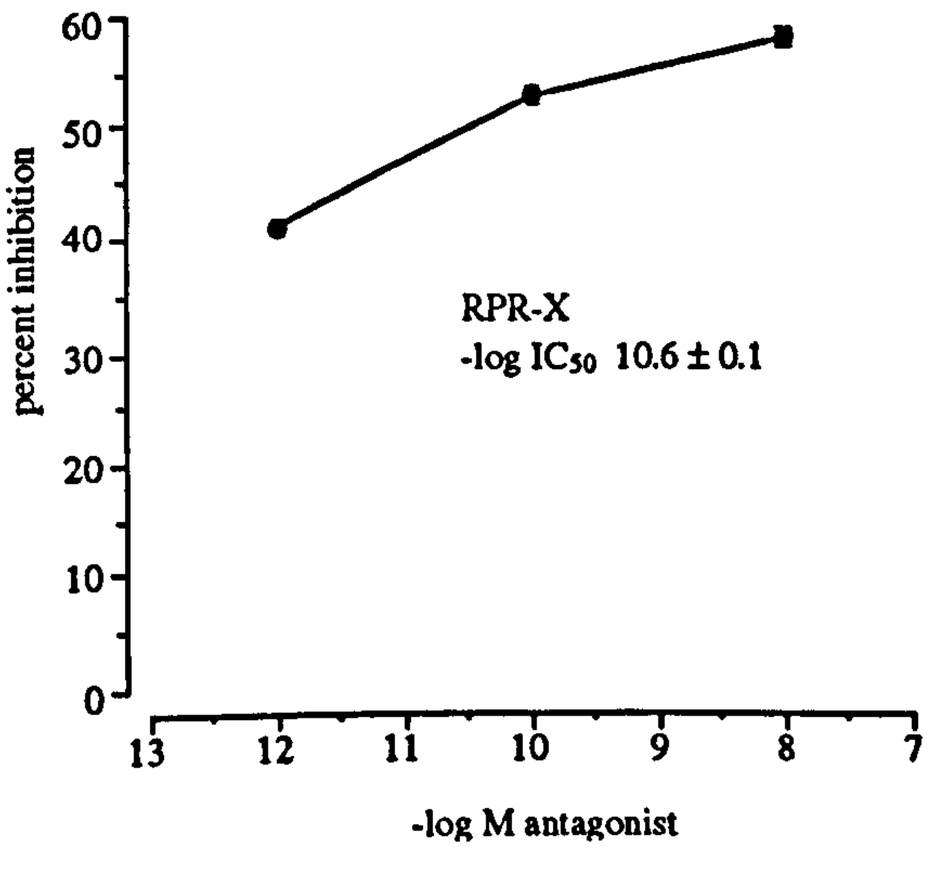
Figure 7.11 Effects of CCK-R antagonists on sCCK-8 stimulated growth of NIH3T3CCK-BR cells in culture. Cells were grown in the presence of various concentrations of antagonists (1 pM, 100 pM and 10 nM) in DMEM containing 100 nM sCCK-8 for 8 days. Each point represents the mean ± s.e. for each group. Two wells of cells were tested for each concentration and each experiment was performed six times (n=12/treatment group). CCK-R antagonists inhibited sCCK-8-stimulated growth in NIH3T3CCK-BR cells.

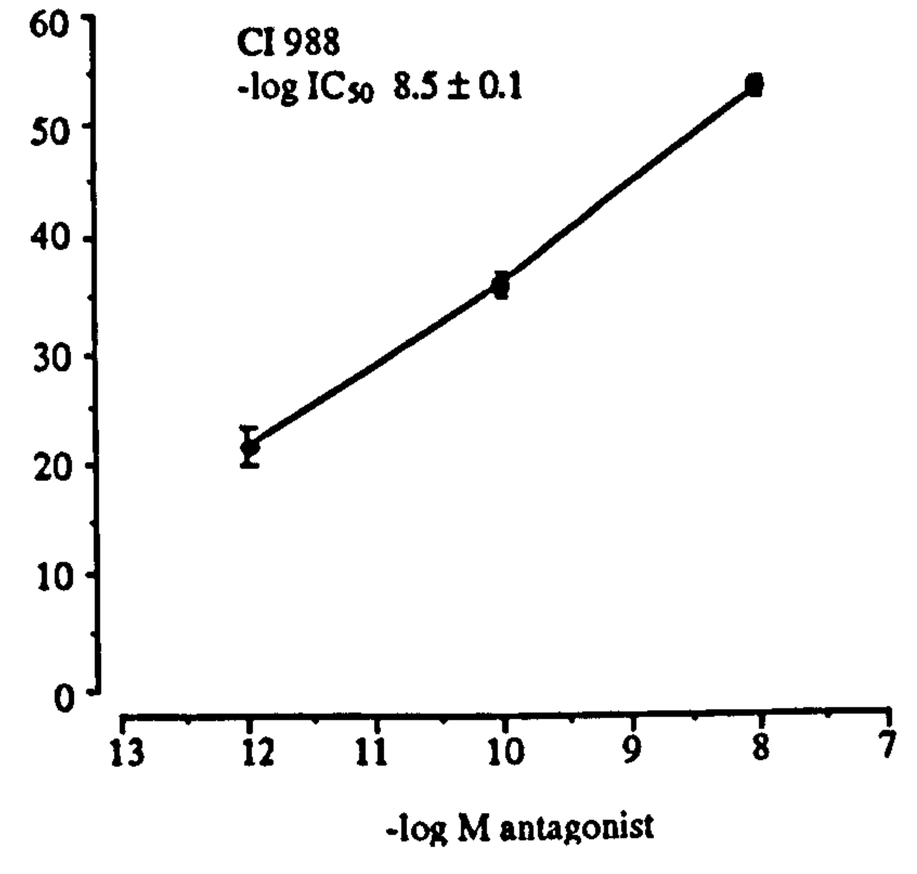

L-364,718
-log IC50 > 8.0
percent inhibition
60
50
40
30
20
10
0
13
12
11
10
9
8
7


CR1409
-log IC50 > 8.0
60
50
40
30
20
10
0
13
12
11
10
9
8
7


L-365,260
-log IC50 8.3 ± 0.1
percent inhibition
60
50
40
30
20
10
0
13
12
11
10
9
8
7


L-740,093
-log IC50 8.6 ± 0.1
60
50
40
30
20
10
0
13
12
11
10
9
8
7


RPR-X
-log IC50 10.6 ± 0.1
percent inhibition
60
50
40
30
20
10
0
13
12
11
10
9
8
7
-log M antagonist


CI 988
-log IC50 8.5 ± 0.1
60
50
40
30
20
10
0
13
12
11
10
9
8
7
-log M antagonist

7.7 Radioligand binding studies in Mia PaCa-2 and NIH3T3CCK-BR cells

7.7.1 *Methods*

The methods are described in section 4.5. Whole cells were incubated for 150 min with the CCK-BR antagonist, [^{3}H]-PD140,376. The bound radioactivity was determined by counting in a Beckman LS6500 liquid scintillation counter. Total and non-specific binding were defined in the absence and presence of L-365,260 respectively.

7.7.2 *Results*

7.7.2.1 *Mia PaCa-2 and NIH3T3CCK-BR cells*

The CCK-BR antagonist bound to both cell types (Figure 7.12A and B). The total and non-specific binding of [^{3}H]-PD140,376 increased as the number of cells increased.

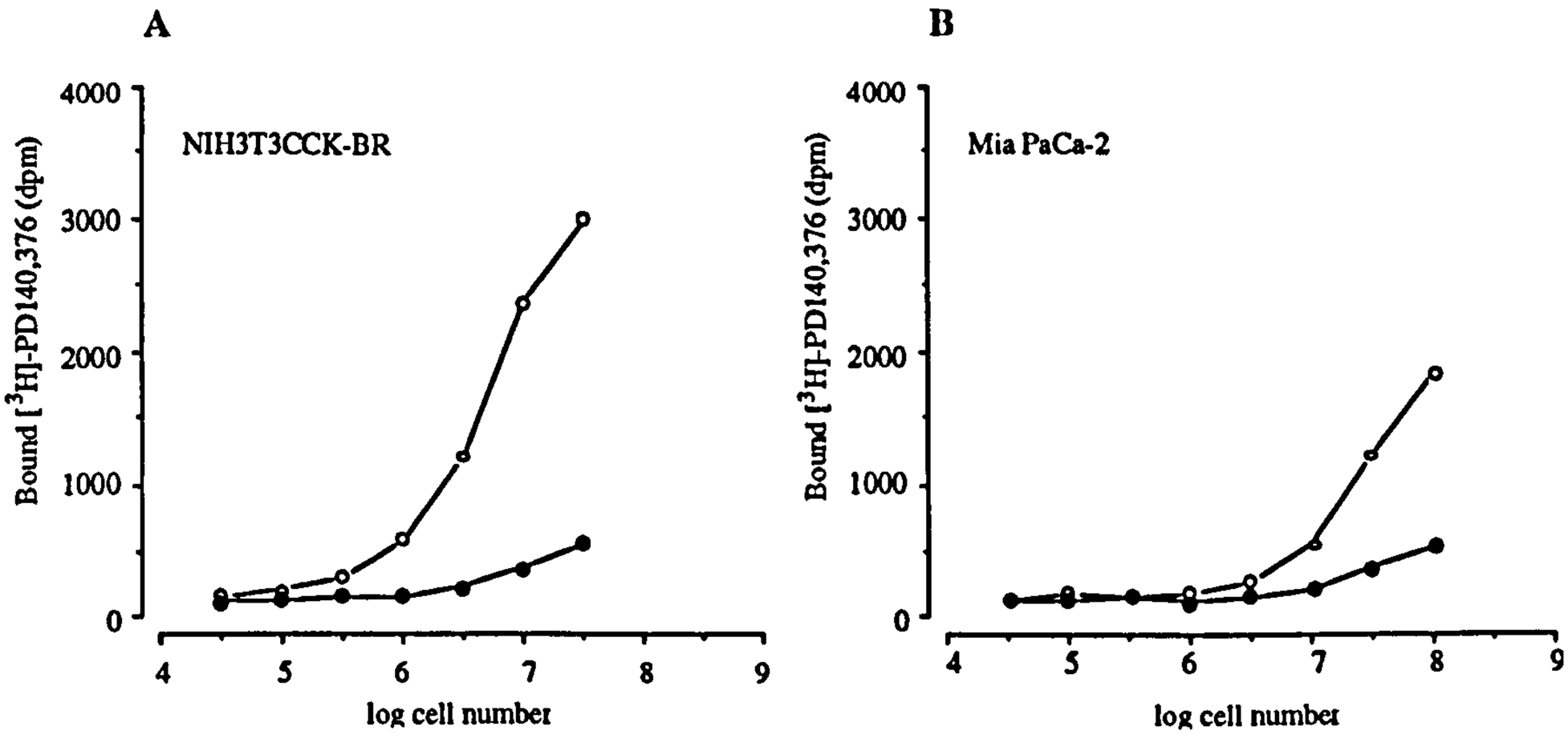


Figure 7.12 Total (○) and non-specific binding (●) of [^{3}H]-PD140,376 to NIH3T3CCK-BR and Mia PaCa-2 cells. Each point represents one experiment carried out in triplicate. Total and non-specific binding of [^{3}H]-PD140,376 increased with an increase in cell number.

The binding of [^{3}H]-PD140,376 increased linearly upto 3 x 10^7 Mia PaCa-2 cells and 3 x 10^6 NIH3T3CCK-BR cells (Figure 7.13). The NIH3T3CCK-BR cells have approximately 12.5 fold more CCK-BR than the Mia PaCa-2 cells, assuming that the affinity of the antagonist for the CCK-BR in both Mia PaCa-2 and NIH3T3CCK-BR is similar.

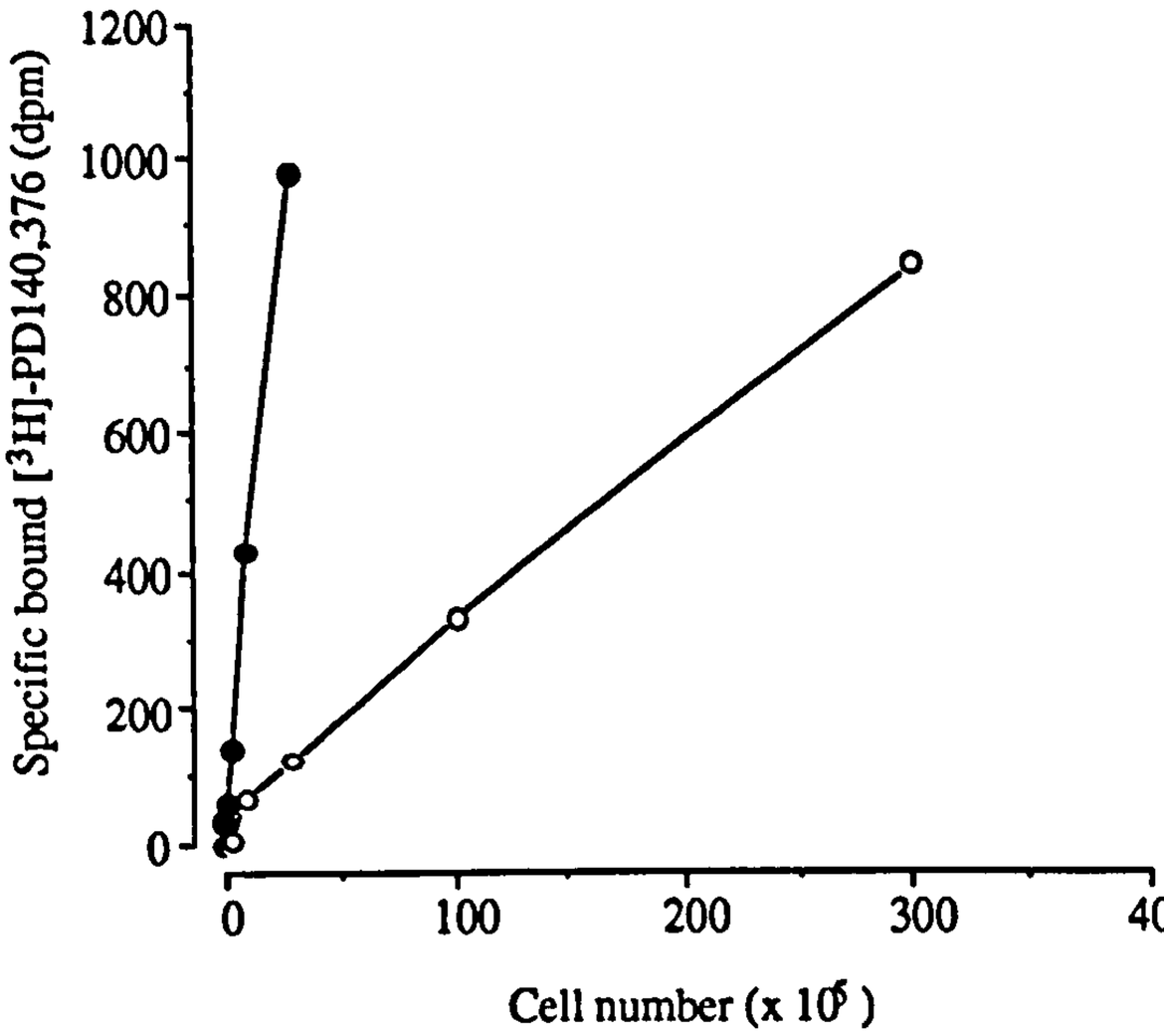


Figure 7.13 Graph comparing the linearity of [^{3}H]-PD140,376 binding to Mia PaCa-2 (○) and NIH3T3CCK-BR cells (●). Each point represents one experiment carried out in triplicate. The binding of [^{3}H]-PD140,376 increased linearly with cell number.

7.8 Molecular studies to determine CCK-R status in human pancreatic cancer cell lines

7.8.1 *Methods*

7.8.1.1 *RT-PCR*

The methods are described in section 4.1. Total RNA was extracted from all the cell lines grown and reverse-transcribed into cDNA. The cDNA was amplified using the CCK-R specific primers using PCR and the amplified products subjected to agarose gel electrophoresis.

7.8.2 *Results*

The two sets of gene specific primers were effective in amplifying either a 340 bp fragment exclusively from the CCK-AR or a 430 bp fragment exclusively from the CCK-BR. Comparison of sequencing studies with published data (de Weerth *et al.*, 1993a and Pisegna *et al.*, 1992a) confirmed that the amplified PCR products were fragments of the CCK-A and CCK-B/gastrin receptor gene.

The RT-PCR showed the expression of both CCK-A and CCK-B/gastrin receptors in all 8 pancreatic tumour cell lines investigated (Capan-1, AsPc-1, BxPc-3, Hs766T, Mia PaCa-2, Panc-1, KPan, Capan-2) as shown in Figure 7.14. A T cell lymphoma cell line which is known to express both CCK receptor types was utilised as a positive control.

7.8.3 *Methods*

7.8.3.1 *RNase protection assay*

The methods are described in section 4.4. Total RNA from each cell line and positive control was hybridised with the radioactively labelled CCK-AR or CCK-BR probe overnight. The unhybridised RNA (single stranded) was digested with RNase and the remaining product electrophoresed on a polyacrylamide gel. The gel was exposed to film for several days (7-12 days) before developing.

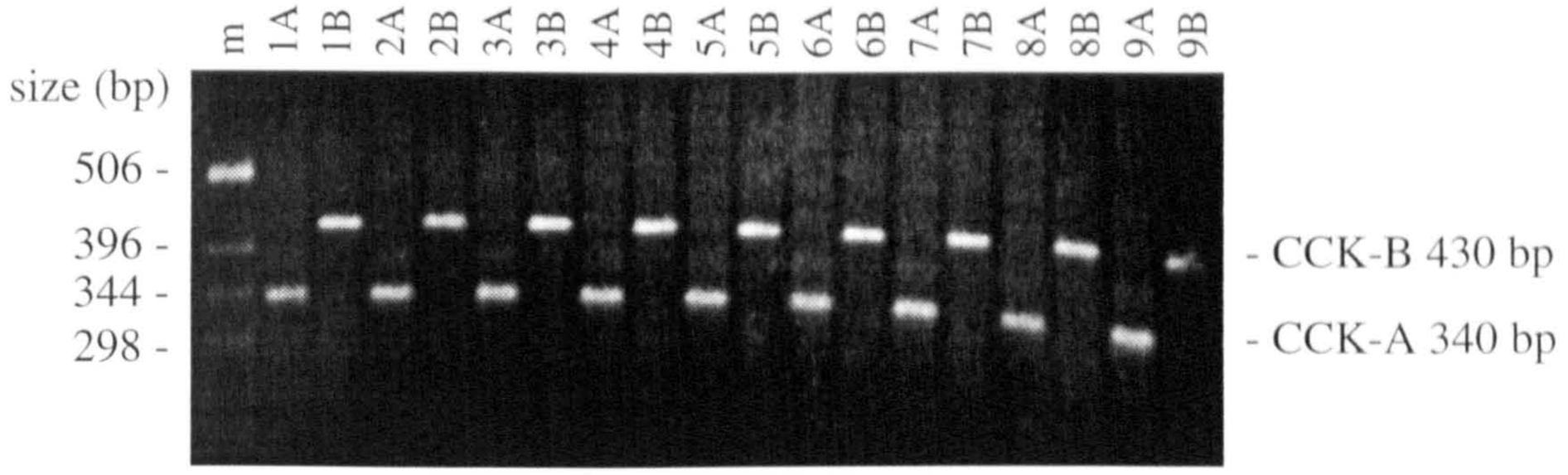


Figure 7.14 Agarose gel electrophoresis of CCK-AR and CCK-BR RT-PCR products in 8 human pancreatic cancer cell lines.

The size of the amplified products were 340 bp and 430 bp for the CCK-A (labelled as A) and CCK-B/gastrin (labelled as B) receptor fragments, respectively. A T-cell lymphoma cell line was used as a positive control in this assay.

Lanes 1A, 1B: Capan-1; 2A, 2B: AsPc-1; 3A, 3B: BxPc-3; 4A, 4B: Hs766T; 5A, 5B: Mia PaCa-2; 6A, 6B: Panc-1; 7A, 7B: KPan; 8A, 8B: Capan-2; 9A, 9B: T-cell line; m: molecular markers.

7.8.4. *Results*

The RNase protection assay revealed a clear band of the expected size for both the protected CCK-A (340 bp) and CCK-B/gastrin (430 bp) receptor mRNA from human gall bladder and stomach mucosa respectively. The expression of both CCK-A and CCK-B/gastrin receptors utilising 50 µg of total RNA was undetectable in all the pancreatic tumour cell lines investigated as shown in Figure 7.15 and 7.16.

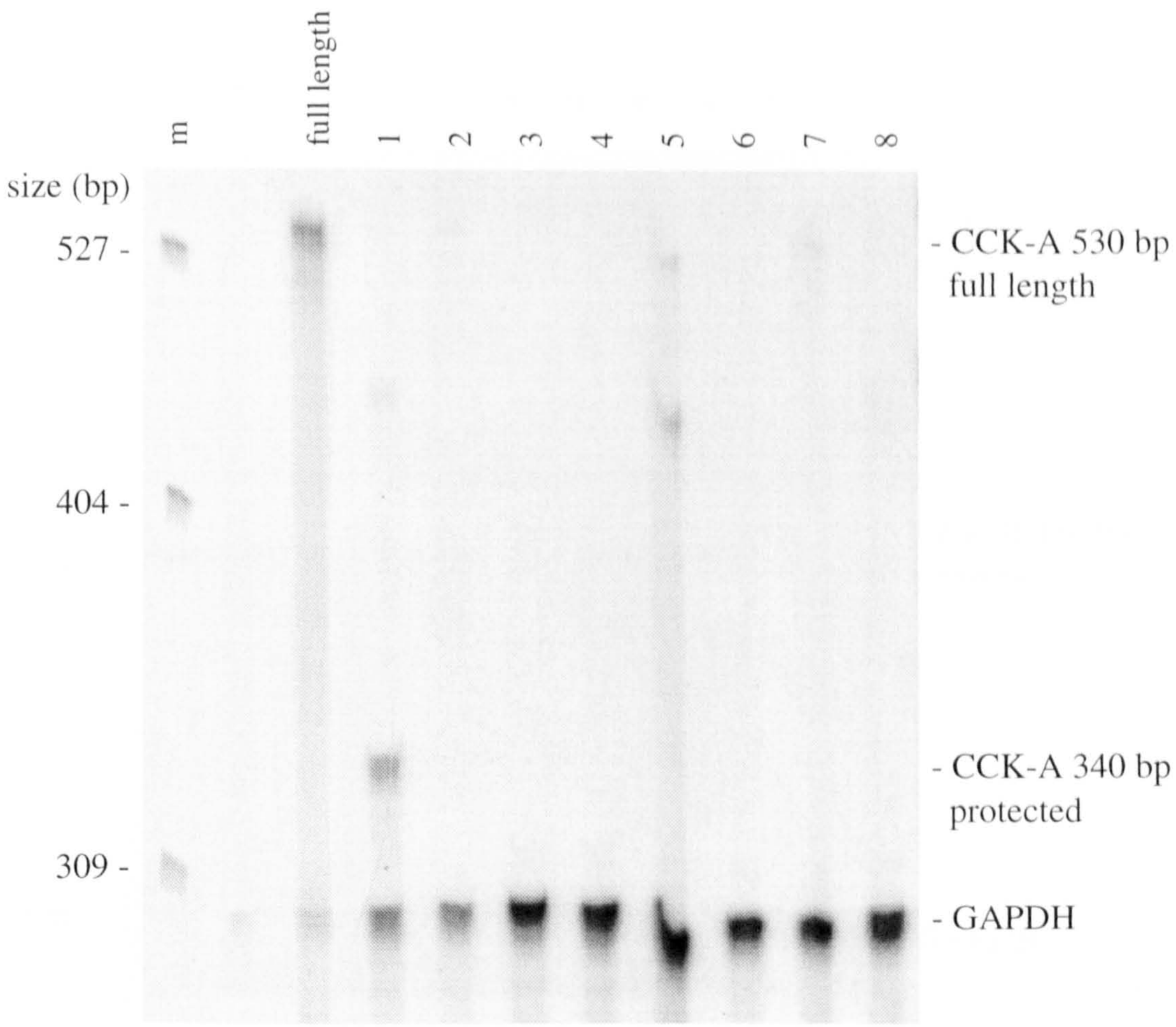


Figure 7.15 Autoradiography of a 6% PAGE gel following the RNP assay.

The CCK-AR mRNA was not detected in 7 human pancreatic tumour cell lines but was present in human gall bladder (lane 1).

The sizes of the full length probe and protected CCK-AR fragment were 530 bp and 340 bp respectively. Human gall bladder (lane 1) was used as positive control for the CCK-A receptor.

Lanes 1: positive control; 2: Capan-1; 3: AsPc-1; 4: BxPc-3; 5: Hs766T; 6: Mia PaCa-2; 7: Panc-1; 8: KPan; m: molecular markers. The protected fragments were resolved on a 6% polyacrylamide gel at 55W for 5 hrs in 1 x TBE buffer.

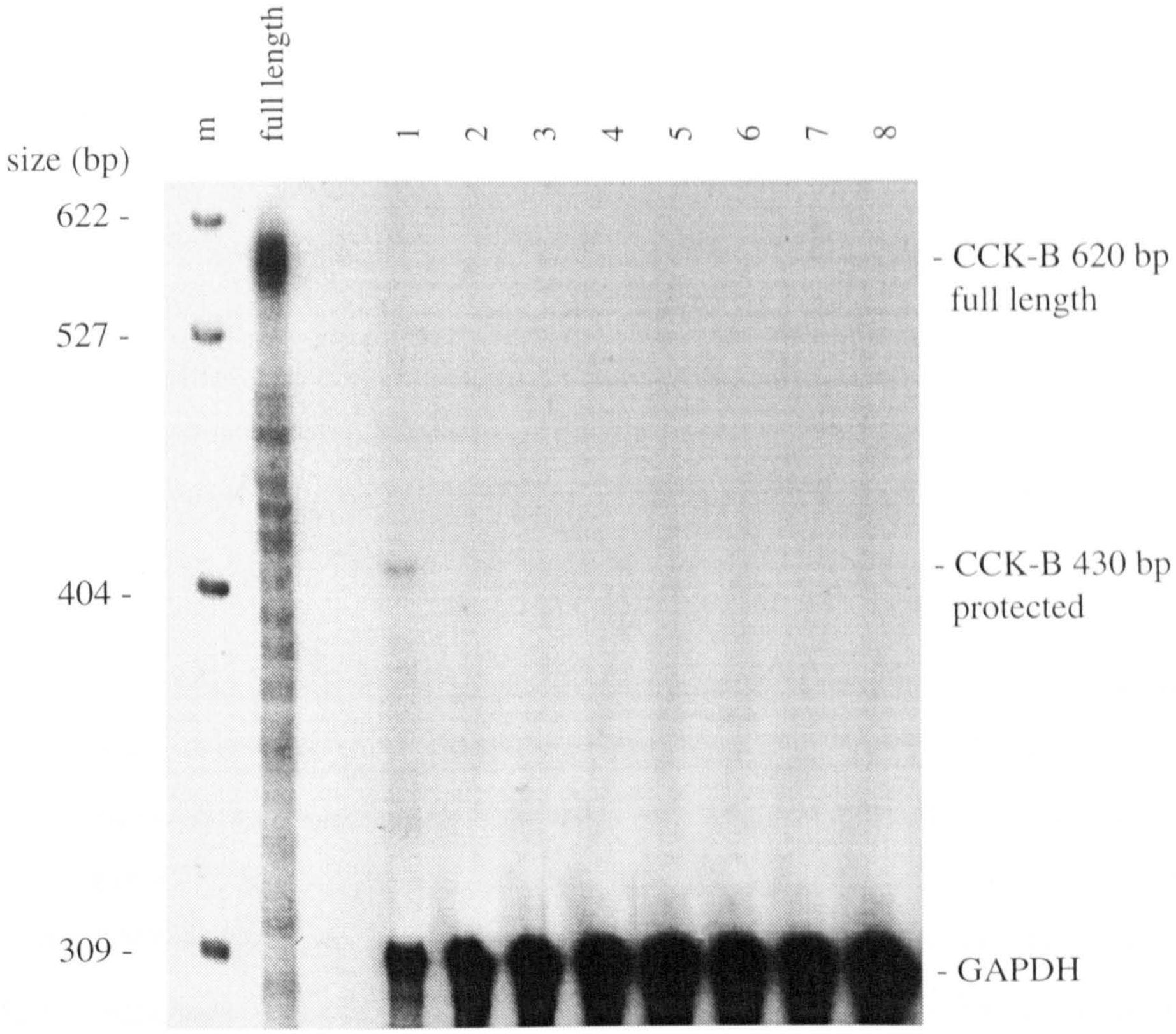


Figure 7.16 Autoradiography of a 6% PAGE gel following the RNP assay.

The CCK-BR mRNA was not detected in 7 human pancreatic tumour cell lines but was present in human stomach mucosa (lane 1).

The sizes of the full length probe and protected CCK-BR fragment were 620 bp and 430 bp respectively. Human stomach mucosa (lane 1) was used as positive control for the CCK-B receptor.

Lanes 1: positive control; 2: Capan-1; 3: AsPc-1; 4: BxPc-3; 5: Hs766T; 6: Mia PaCa-2; 7: Panc-1; 8: KPan; m: molecular markers. The protected fragments were resolved on a 6% polyacrylamide gel at 55W for 5 hrs in 1 x TBE buffer.

7.9 Discussion

The 7 human pancreatic cancer cell lines (Capan-1, AsPc-1, BxPc-3, Hs766T, Mia PaCa-2, Panc-1, KPan) expressed both CCK-A and CCK-B receptors as detected by RT-PCR. However, the RNase protection assay (utilising 50 µg of RNA) was not sufficiently sensitive to detect the levels of CCK-R expression. Demonstration of receptor mRNA by RT-PCR alone does not always reflect the presence of functional receptor proteins in the cell membrane, since theoretically only one molecule of mRNA is needed for detection by RT-PCR. The RNP assay is the most sensitive quantitative method known to date to detect low levels of message expression.

The NIH3T3CCK-BR cells were originally used to study CCK-BR signalling transduction pathways by Dr. Matsui (see Taniguchi *et al.*, 1994). Thus, these mouse fibroblasts have been artificially transfected with a high proportion of the human CCK-BR. The CCK-B receptor was detected by RT-PCR as well as by RNP assay in the NIH3T3CCK-BR cells. Furthermore, the radioligand binding assay, with the CCK-BR antagonist [^{3}H]-PD140,376 showed the NIH3T3CCK-BR cells to have approximately 12.5 fold more CCK-BR than the Mia PaCa-2 cells. This 12.5 fold difference may be a critical difference in the sensitivity limit of the RNP assay. Thus from the molecular (see Figure 9.4 in Chapter 9) and binding studies in NIH3T3CCK-BR cells, one can assume that RNP is indicative of a certain density of functional receptors that can result in a biological response. Moreover, the cell lines Mia PaCa-2 and BxPc-3 were unresponsive to both sCCK-8 and nsG-17 in proliferation studies *in vitro*. This finding can best be explained by the lack of sufficient CCK-A or CCK-B/gastrin receptors (as indicated by RNP assay) to elicit a trophic response.

In vitro published studies on the trophic effects of exogenous CCK have given rise to conflicting results. Studies on pancreatic tumour cell lines show either stimulation, inhibition or no effect. Funakoshi & Kono, (1992) found that CCK stimulated growth of the human pancreatic cancer cell line KP-1N, while Morimoto *et al.* (1993) observed that CCK-8 increased the uptake of [^{3}H]-thymidine in only one pancreatic tumour cell

line (PC-F3) out of five. Smith *et al.* (1991) reported trophic responses to CCK-8 or CCK-9 analogs in the cell lines Mia PaCa-2, BxPc-3, Capan-2 and Panc-1. However, other experiments have shown no effect of these peptides on tumour cell growth *in vitro* and some studies have even shown inhibition (Morimoto *et al.*, 1993; Liehr *et al.*, 1990; Nio *et al.*, 1993). Liehr *et al.* (1990) observed no change in DNA, protein or cloning efficiency in Panc-1 and Mia PaCa-2 cells after incubation with CCK-8 or a CCK-9 analog. Nio *et al.* (1993) observed an inhibitory effect in *in vitro* DNA synthesis by CCK-8 in 2 pancreatic cancer cell lines, PC-YY and PC-T1. Robertson *et al.* (1995), have also shown a lack of effect of G-17 on Mia PaCa-2 cell growth and this correlated with an absence of [^{125}I]iodo tyrosyl G-17 binding sites. Our finding of very low expression of mRNA for both CCK receptor-types, indicating a low CCK-A and CCK-B/gastrin receptor population, are consistent with a poor trophic response in these cell lines to CCK or gastrin.

Smith *et al.* (1995) have shown that gastrin-17 had a proliferative effect on the pancreatic cancer cell lines, Panc-1, BxPc-3, MDA-Panc-28 and MDA-Amp-7 and that the gastrin-induced proliferation was inhibited by L-365,260 (CCK-BR antagonist) and not L-364,718 (CCK-AR). As mentioned earlier we found a lack of response of BxPc-3 to nsG-17 and sCCK-8 used at a range of concentrations. A possible explanation for the differential findings is that the cells in our laboratory are at a different passage number, hence displaying different characteristics. It has been found that receptors can be lost upon passaging of cell lines *in vitro* and in the passaging of xenografted tumours (Singh *et al.*, 1991). However, no molecular or binding studies were carried out by this group in order to confirm the extent and presence of the receptors for comparison.

CCK receptor binding studies in homogenised human pancreatic tumours and tumour-derived cell lines have also given conflicting results (Singh *et al.*, 1991; Smith *et al.*, 1993, 1994). Smith and co-workers (1992) have reported a binding site of CCK in the nuclear fraction and minimal or no binding to the membranes of the Panc-1 cell line. However, further work on this same cell line from the same laboratory reported CCK-

R in the membrane fraction and not in the nucleus (Smith *et al.*, 1994). Membrane-bound CCK-BR have also been reported in cell lines Mia PaCa-2, BxPc-3 and Capan-1, but no data have been published on CCK binding in whole cells by this group. Singh *et al.* (1991) found no CCK-R in whole Mia PaCa-2 cells using ligand binding assays. These conflicting results make it important to determine initially the receptor status of the cell lines being investigated using well developed techniques.

Both sCCK-8 and nsG-17 induced the growth of NIH3T3CCK-BR cells *in vitro*. The agonists had equal affinity for the CCK-BR. It is well documented in published literature that both sulphated and non-sulphated analogues of cholecystokinin and gastrin have similar affinities for the CCK-BR (Saito *et al.*, 1981).

Comparison of the results between the various antagonists correlate well with published data (see Table 7.2). All the CCK-BR antagonists inhibited CCK-BR stimulated growth in the nanomolar range. The rank order of potency of the antagonists for the CCK-BR are in agreement with work carried out by Saito *et al.* (1981), Woodruff & Hughes (1991), and Showell *et al.* (1994). The three groups measured the displacement of CCK-8 from the rat pancreas CCK-AR, and the mouse/guinea pig cortex CCK-BR.

The CCK-AR specific antagonists L-364,718 (devazepide) and CR 1409 had IC_{50} values greater than 10 nM. This result is not surprising as these antagonists are not known to act on CCK-BR at these concentrations. Woodruff and Hughes (1991) showed that the IC_{50} value of CR 1409 was greater than that of L-364,718 for the mouse cortex CCK-B binding sites, indicating that CR 1409 is less likely to bind to the CCK-BR than L-364,718. This finding indirectly confirms our results since 10 nM of L-364,718 had a greater inhibitory effect on the sCCK-8 induced growth response than CR 1409.

More importantly the results show that these CCK-BR antagonists are capable of inhibiting CCK-BR induced cell growth *in vitro* as shown in the NIH3T3CCK-BR cells.

Antagonist (receptor-type)	Rat pancreas IC_{50} (nM)	Mouse cortex IC_{50} (nM)	Reference
L-364,718 (A)	0.2	31 (>10)	Woodruff & Hughes, 1991
CR 1409 (A)	130	3×10^5 (>10)	Saito *et al.*, 1981
CI988 (B)	2717	1.7 (4)	Woodruff & Hughes, 1991
L-365,260 (B)	240	5.2 (5.7)	Woodruff & Hughes, 1991
L-365,260 (B)	736	8.5 (5.7)	Showell *et al.*, 1994
L-740,093 (B)	1604	0.1 (3.3)	Showell *et al.*, 1994

	Rat pancreas IC_{50} (nM)	Guinea pig cortex IC_{50} (nM)	

Table 7.2 Showing selective CCK-R antagonist receptor affinities. Values in brackets obtained in NIH3T3CCK-BR by Mandair *et al.*, 1997.

The CCK-R antagonists had no effect on the basal growth of the pancreatic cancer cell lines, Mia PaCa-2 or BxPc-3. These results indicate that the CCK-AR and CCK-BR are not involved in an autocrine manner in these cancer cells *in vitro*. Our molecular studies confirm this data and support the view that there are not enough CCK-R expressed in these cells to play a role in cell growth *in vitro*.

Recently, Smith *et al*. (1996) have shown that the growth of the human pancreatic cancer cell line, BxPc-3 is tonically stimulated by the autocrine production of gastrin. They showed this by incubating the BxPc-3 cells in serum-free medium in the presence of L-365,260. The results obtained in our laboratory are contradictory to this finding. The concentration of the antagonist L-365,260 that significantly inhibited the growth of the BxPc-3 cells in serum-free medium by this group was 100 μM. This concentration was toxic to the BxPc-3 cells in our laboratory. Thus, their results may be explained by the toxicity of the antagonist at these high concentrations. Another possible explanation (as mentioned previously) for the difference in results is that the cells in our laboratory

may be at a different passage number, hence displaying different characteristics. The BxPc-3 cells in serum-free medium had a doubling time of 3 days and 10 days respectively in Smith's laboratory and our laboratory which is indicative of differences in cell characteristics. It has also been shown that receptors can be lost upon passaging of cell lines *in vitro* and in the passaging of xenografted tumours (Singh *et al.*, 1991) which may also explain the differences in the results.

In 1993, Morimoto and co-workers showed no effect of devazepide on the basal growth of the human pancreatic cancer cell line, PC-HN. Later Seva *et al.* (1994) reported the lack of effect of the CCK-BR antagonists, L-365,260 and PD-134308 on the basal proliferation of the rat pancreatic cancer cell line, AR42J. However autocrine growth regulation has been shown to be important in other human cancers such as colon cancer (Hoosein *et al.*, 1990) and small cell lung cancer (Rehfeld *et al.*, 1989).

This work highlights the importance of (1) characterisation of human pancreatic cancer cell lines irrespective of published data (2) pancreatic tumour cell lines as a poor model of the primary tumour.

CHAPTER 8

K-*RAS* STATUS AND FAK PHOSPHORYLATION IN ESTABLISHED HUMAN PANCREATIC CANCER CELL LINES

CHAPTER 8

8.1 Background

Cancer is a disease with many different kinds of genetic alterations which may evolve from simple or more complex types and numbers of mutations within the same neoplastic cell (Aldaz *et al.*, 1987, Bremner & Balmain, 1990). Among the most frequent genetic changes in human pancreatic cancer are point mutations of the K-*ras* proto-oncogene (K-*ras*, Smit *et al.*, 1988; Grunewald *et al.*, 1989; Lemoine *et al.*, 1992b). K-*ras* mutations occur in pancreatic cancer almost exclusively on codon 12 (Almoguera *et al.*, 1988) resulting in a constitutively active ras protein. There is increasing evidence that most GPCR such as the CCK-BR (Seva *et al.*, 1996) feed through the ras protein upon activation. In late 1994, Taniguchi and co-workers reported that the stimulation of the CCK-BR expressed in transfected mouse fibroblasts (NIH3T3CCK-BR) resulted in the phosphorylation of a kinase called focal adhesion kinase (FAK). FAK phosphorylation is independent of ras but is thought to be dependent on another small G protein, rho (Secki *et al.*, 1995). In view of this literature we investigated the effects of cholecystokinin and gastrin upon the phosphorylation of FAK, a more immediate event upon receptor stimulation, by-passing the effects of constitutively active ras.

8.2 Aims

The aims of this study were i) to determine the K-ras status in the established human pancreatic cancer cell lines; ii) to investigate the effect of sCCK-8 and nsG-17 on tyrosine phosphorylation of FAK in Mia PaCa-2 cells and iii) to determine the effects of various CCK-R antagonists on FAK tyrosine phosphorylation in Mia PaCa-2 cell line using the NIH3T3CCK-BR as a control.

8.3 The K-ras status in established human pancreatic cancer cell lines

8.3.1 *Methods*

8.3.1.1 *SSCP*

The methods are described in section 4.6.4. Single strand conformation polymorphism (SSCP) was carried out by the amplification of K-*ras* exon 1 from genomic DNA from each cell line in the presence of α-[^{32}P]deoxy-cytidine triphosphate. The amplified products were subjected to polyacrylamide gel electrophoresis. Autoradiography film was exposed to the gel overnight and developed. The PCR products of K-*ras* exons 1 and 2 were cloned and sequenced as in methods section 4.6. Autoradiography film was exposed to the gel overnight and developed.

8.3.2 *Results*

The PCR of K-*ras* exon 1 and exon 2 resulted in fragments of 120 bp and 111 bp respectively, as shown in Figures 8.1 and 8.2. The SSCP data showed genetic variability in exon 1 of the K-*ras* gene in cell lines Capan-1, AsPc-1, Mia PaCa-2, Panc-1 and Kpan when compared with the normal pancreas (Figure 8.3). The cloning and sequencing results of the K-*ras* PCR products are summarised in Table 8.1. In 2 of these cell lines, Capan-1 and Mia PaCa-2, there was a substitution from the wild type glycine to a valine in exon 1 at codon 12 as indicated by a base point mutation from guanine (G) to a thymine (T). The remaining 3 cell lines, AsPc-1, Panc-1 and KPan showed a substitution from glycine to aspartate due to a point mutation from guanine (G) to adenine (A). Figure 8.4 shows the various point mutations detected in *K-ras* exon 1 in the pancreatic cancer cell lines examined. There was no mutation detected in the hot spot region of codon 61 in exon 2 in the cell lines investigated (Figure 8.5).

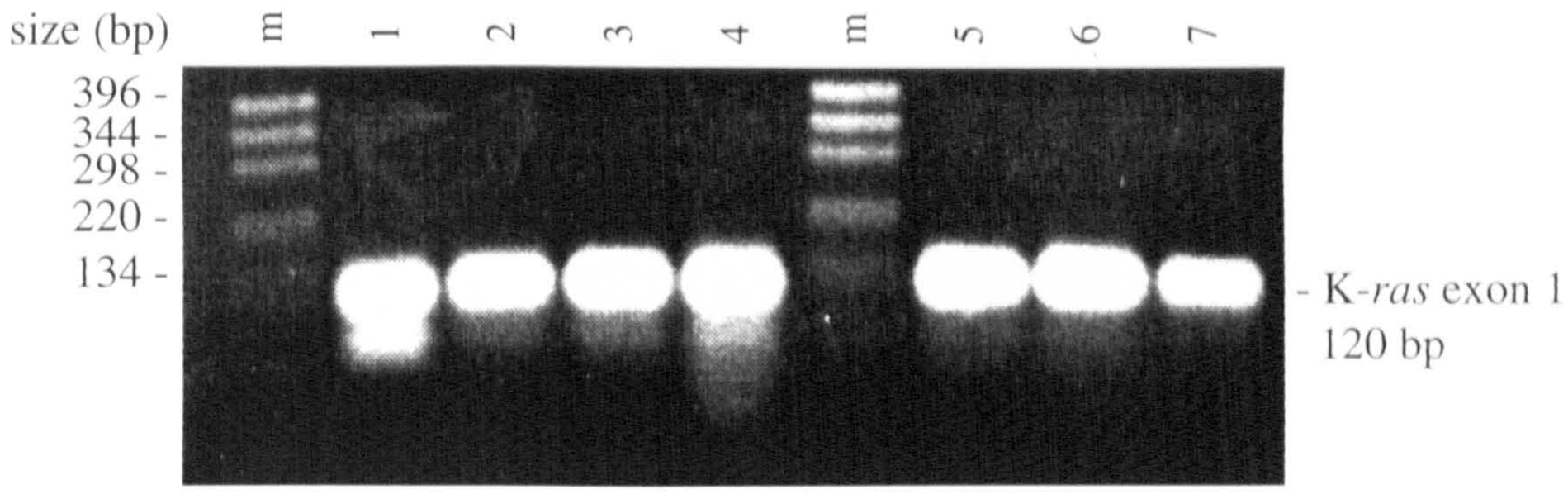


Figure 8.1 Agarose gel electrophoresis of amplified K-*ras* exon 1, 120 bp product. The PCR product was amplified from human pancreatic cancer cell line genomic DNA.

Lanes 1: Capan-1; 2: AsPc-1; 3: BxPc-3; 4: Hs766T; 5: Mia PaCa-2; 6: Panc-1; 7: KPan; m: molecular markers.

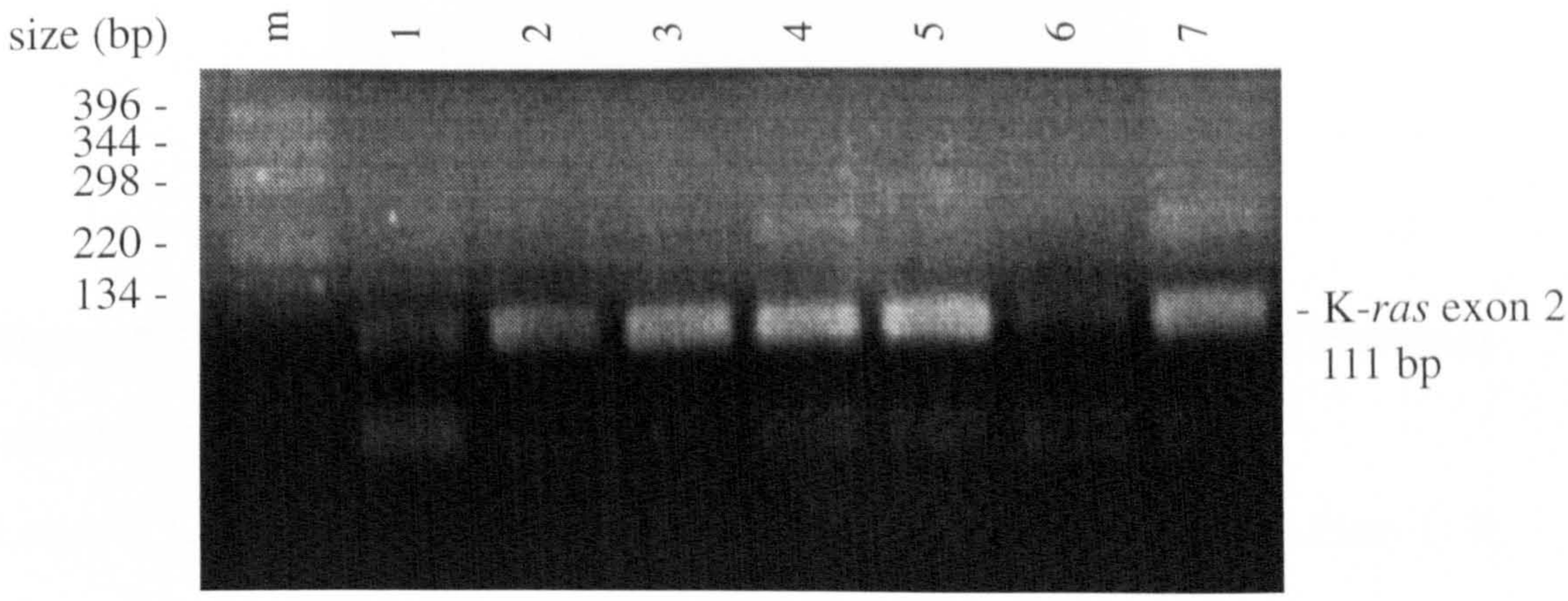


Figure 8.2 Agarose gel electrophoresis of amplified K-*ras* exon 2, 111 bp product. The PCR product was amplified from human pancreatic cancer cell line genomic DNA.

Lanes 1: Capan-1; 2: AsPc-1; 3: BxPc-3; 4: Hs766T; 5: Mia PaCa-2; 6: Panc-1; 7: KPan; m: molecular markers.

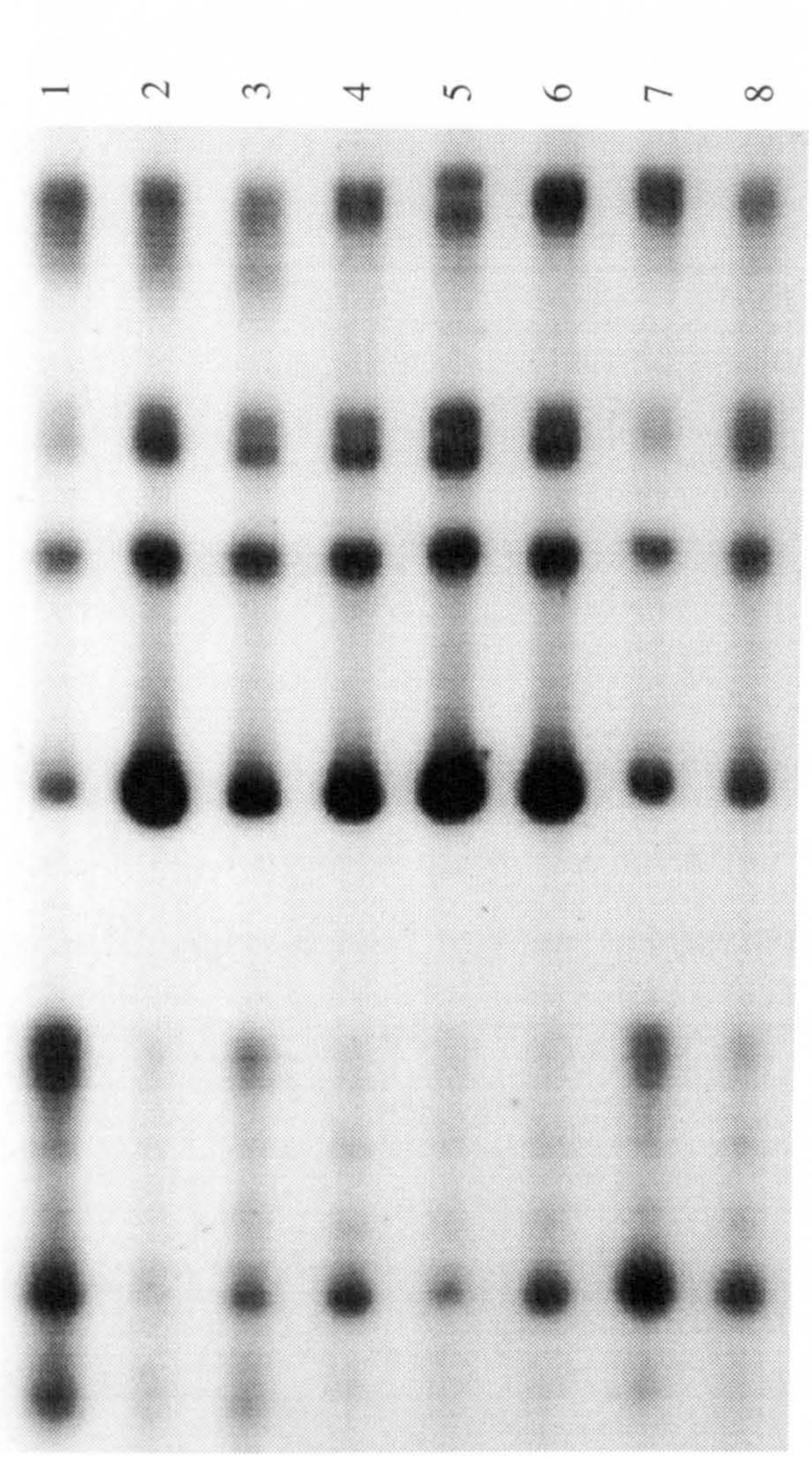


Figure 8.3 Autoradiography of 6% PAGE gel with representative SSCP analysis of exon 1 of K-*ras* gene in 7 human pancreatic tumour cell lines.

Lanes 1: Capan-1; 2: AsPc-1; 3: BxPc-3; 4: Hs766T; 5: Mia PaCa-2; 6: Panc-1; 7: KPan; 8: normal human pancreas.

Electrophoresis conditions shown are 6% nondenaturing polyacrylamide with 10% glycerol and 1.0 x TBE buffer at 4°C.

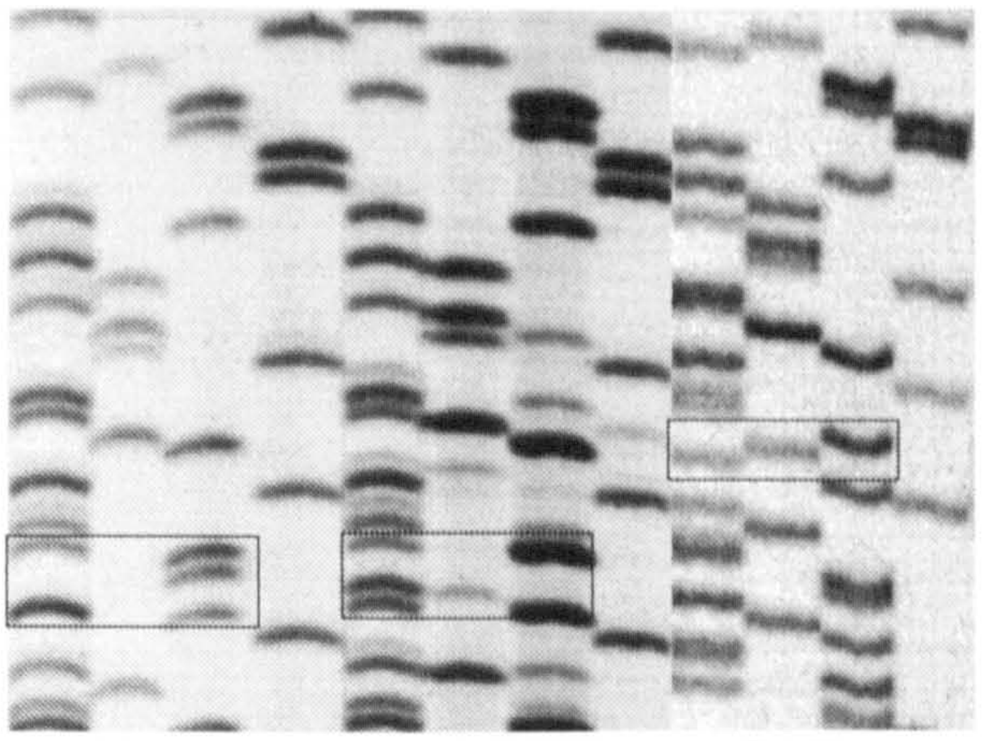


Figure 8.4 Sequences of the K-*ras* gene exon 1 in codon region 11-18 in human pancreatic cancer cell lines. 2 different types of mutations were detected. The sequence of the codon 12 wild type GGT (glycine: central lanes) was mutated to GTT (valine: left lanes) and GAT (aspartate: right lanes). The boxes enclose the codon 12 triplet.

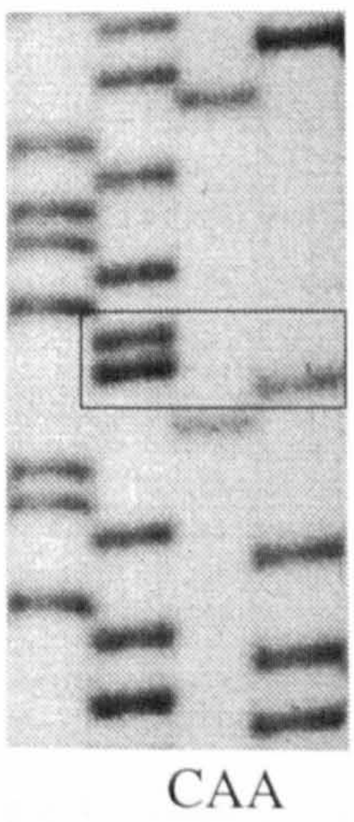


Figure 8.5 Sequences of the K-*ras* gene exon 2 in codon region 58-64 in human pancreatic cancer cell lines. Mutations were undetected. The sequence of the codon 61 wild type CAA (glutamine) is shown. The box encloses the codon 61 triplet.

Tumour Passage	K-*ras* Exon 1 codon 12	K-*ras* Exon 2 codon 61
Capan-1*	G<u>T</u>T	CAA
AsPc-1*	G<u>A</u>T	CAA
BxPc-3	GGT	CAA
Hs766T	GGT	CAA
Mia PaCa-2*	G<u>T</u>T	CAA
Panc-1*	G<u>A</u>T	CAA
Kpan*	G<u>A</u>T	CAA

Table 8.1 Summary of K-*ras* mutations in human pancreatic cancer cell lines. The point mutations in exon 1 at codon 12 are underlined. GGT: glycine, GAT: aspartate, GTT: valine, CAA: glutamine. Five of these tumour cell lines* possess a point mutation in codon 12 of the K-*ras* gene. No mutations were found at codon 61.

8.4 Effects of sCCK-8 and nsG-17 on FAK phosphorylation

8.4.1 *Methods*

The methods are described in Chapter 3. Mia PaCa-2 and NIH3T3CCK-BR cells were stimulated with either sCCK-8 or nsG-17 (section 3.1), lysed (section 3.2) and FAK was immunoprecipitated and positive samples electrophoresed as described in sections 3.3-3.7, transferred to a membrane and immunoblotted with appropriate antibodies (section 3.8-3.10).

8.4.2 *Results*

Phosphorylation studies showed tyrosine phosphorylation of the 125 kDa focal adhesion kinase ($p125^{FAK}$) in the NIH3T3CCK-BR cells (Figure 8.6). The phosphorylation was promoted by both 100 nM sCCK-8 and 100 nM nsG-17.

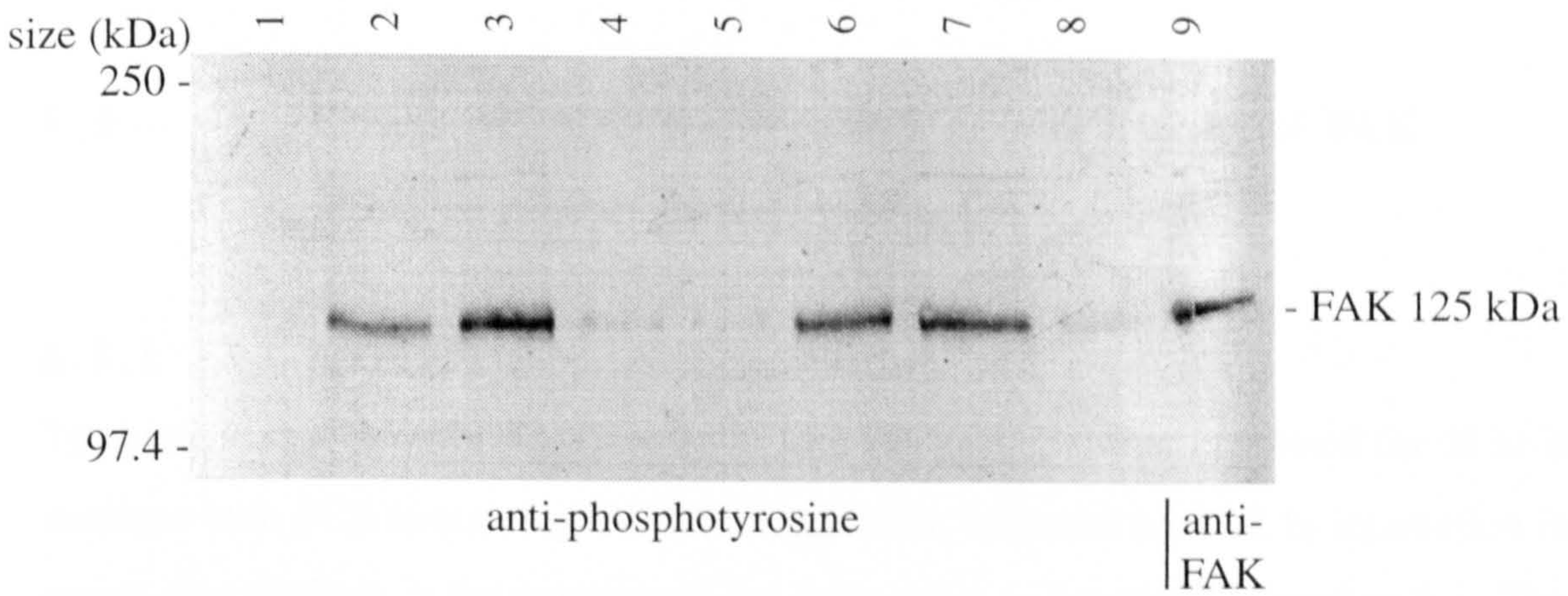


Figure 8.6 Immunoblot of 6.5% PAGE gel with FAK tyrosine phosphorylation in NIH3T3CCK-BR cells induced by sCCK-8 and nsG-17.

NIH3T3CCK-BR FAK immunoprecipitates: Lanes 1+5: untreated; 2+6: 100 nM sCCK-8; 3+7: 100 nM nsG-17; 4+8: 10% bovine calf serum; 9: FAK control cell lysate.

Lanes 1-8 and 9 were immunoblotted with phosphotyrosine and FAK antibodies respectively.

In unstimulated NIH3T3CCK-BR cells the level of FAK phosphorylation was undetectable compared to the stimulated cells. In contrast Mia PaCa-2 cells constitutively expressed phosphorylated FAK. There was no increase in FAK phosphorylation by either nsG-17 or sCCK-8 at the various concentrations in serum-starved Mia PaCa-2 cells (Figure 8.7 and 8.8 respectively). Figure 8.9 shows an elevation in the basal level of phosphorylated FAK in Mia PaCa-2 cells compared with phosphorylated FAK in NIH3T3CCK-BR cells.

8.5 Effects of CCK-R antagonists on elevated basal FAK phosphorylation levels in Mia PaCa-2 cells

8.5.1 *Methods*

The methods are described in Chapter 3. Mia PaCa-2 cells were incubated for 48 hr in medium with FCS containing CCK-R antagonists, followed by a 48 hr incubation in serum-free medium in the presence of the antagonists as described in section 3.1. The cells were lysed and FAK immunoprecipitated (sections 3.2-3.5). The immunoprecipitated samples were electrophoresed, transferred to a membrane and immunoblotted with appropriate antibodies as described in sections 3.6-3.10. The level of FAK phosphorylation in the presence of specific CCK-BR antagonists was determined. The CCK-BR antagonists were chosen due to their inhibitory effect on sCCK-8 induced growth responses in NIH3T3CCK-BR described in chapter 7.

8.5.2 *Results*

The CCK-BR antagonists, RPR-X, L-740,093, L-365,260 and CI 988 did not reduce the elevated basal FAK phosphorylation levels in Mia PaCa-2 cells as shown in Figures 8.10 and 8.11.

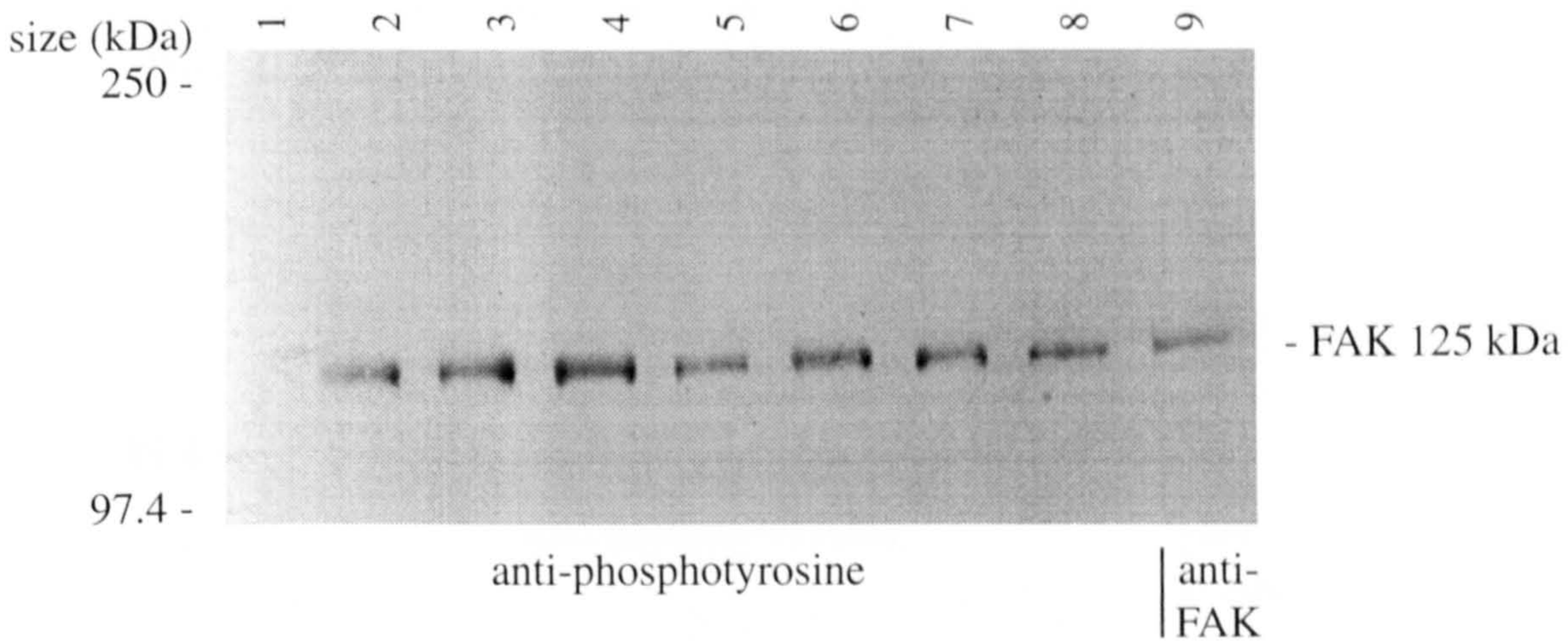


Figure 8.7 Immunoblot of 6.5% PAGE gel showing lack of elevation of FAK tyrosine phosphorylation in Mia PaCa-2 cells with varying nsG-17 concentrations. Mia PaCa-2 FAK immunoprecipitates from lane 1: negative control (beads only); 2: untreated; 3: 500 nM nsG-17; 4: 200 nM nsG-17; 5: 50 nM nsG-17; 6: 10 nM nsG-17; 7: vehicle control; 8: positive control (endothelial cell lysate); 9: FAK control. Lanes 1-8 and 9 were immunoblotted with phosphotyrosine and FAK antibodies respectively.

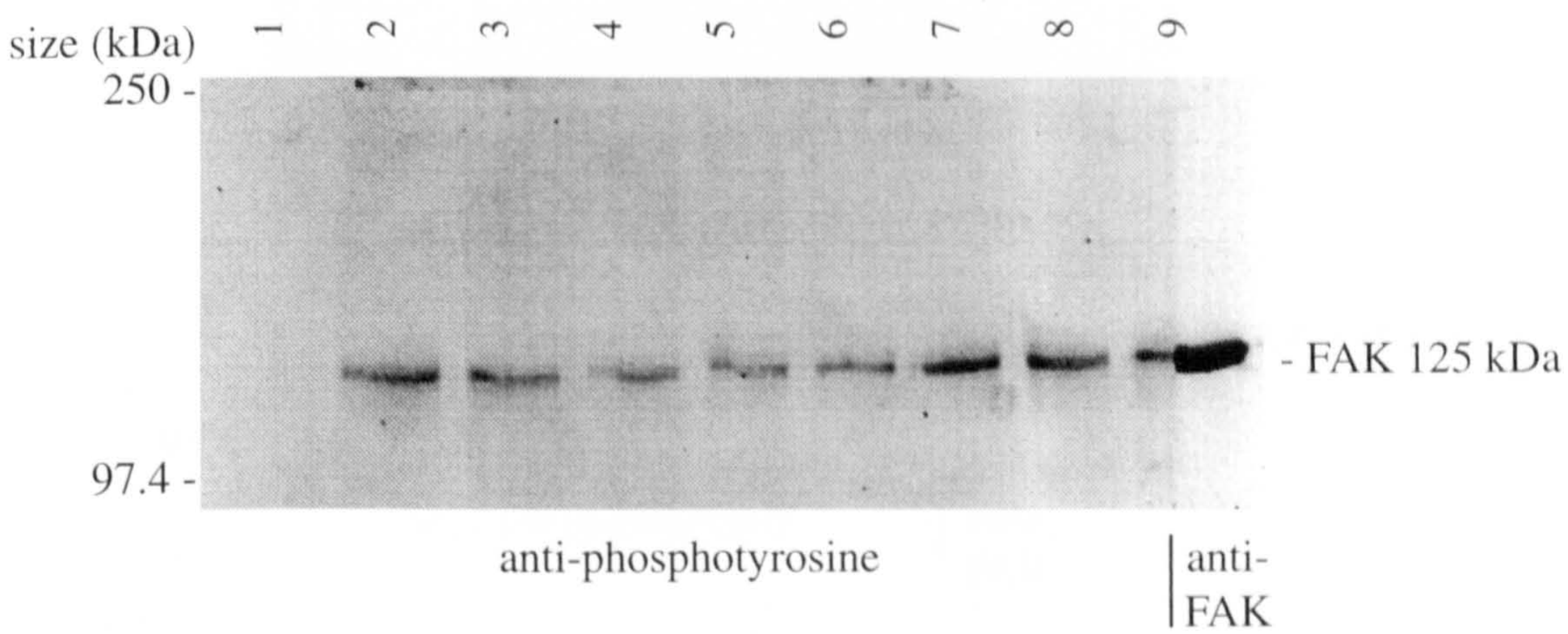


Figure 8.8 Immunoblot of 6.5% PAGE gel showing lack of elevation of FAK tyrosine phosphorylation in Mia PaCa-2 cells with varying sCCK-8 concentrations. Mia PaCa-2 FAK immunoprecipitates from lane 1: negative control (beads only); 2: untreated; 3: 500 nM sCCK-8; 4: 200 nM sCCK-8; 5: 50 nM sCCK-8 6: 10 nM sCCK-8; 7: vehicle control; 8: positive control (endothelial cell lysate); 9: FAK control.

Lanes 1-8 and 9 were immunoblotted with phosphotyrosine and FAK antibodies respectively.

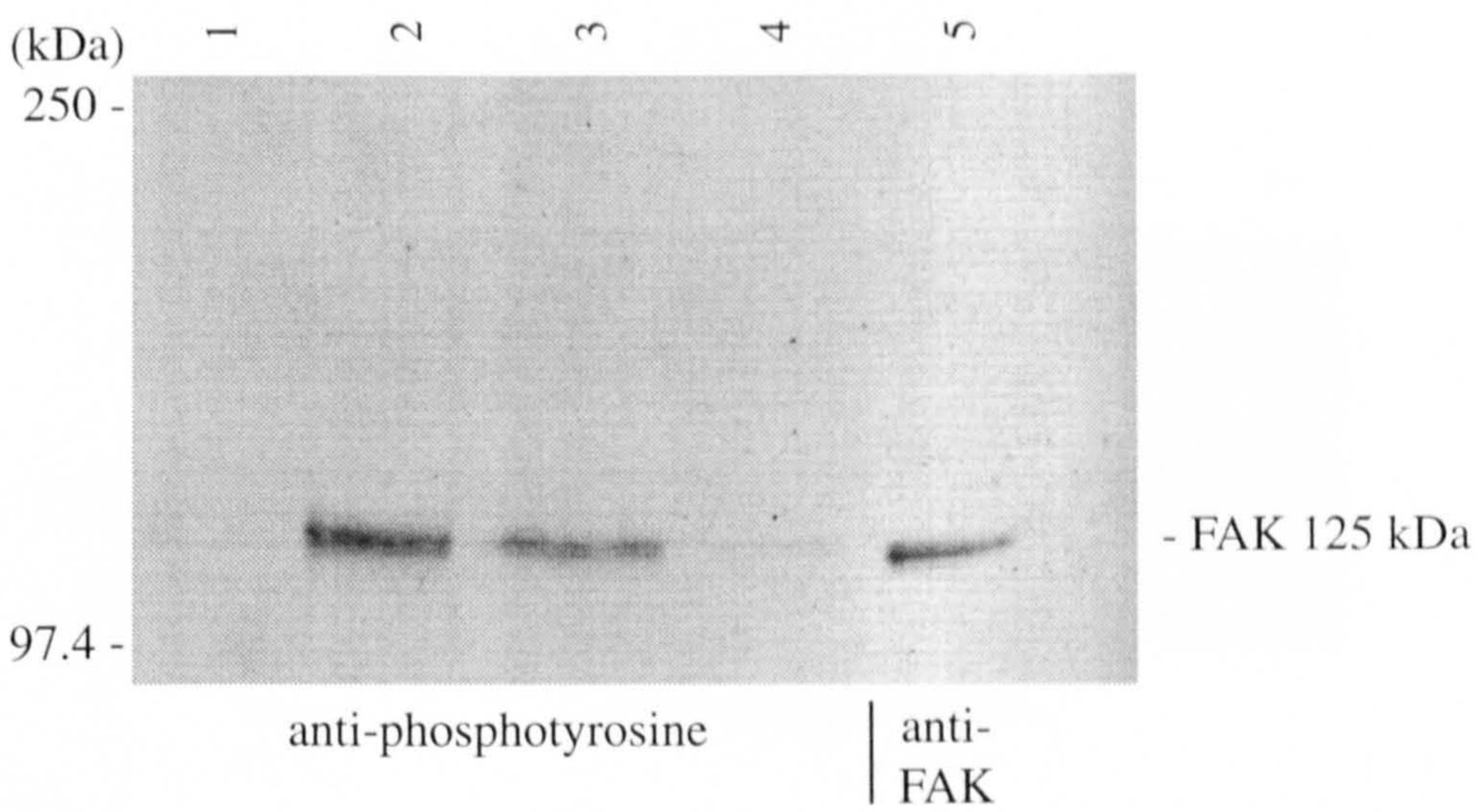


Figure 8.9 Immunoblot of 6.5% PAGE gel showing basal FAK tyrosine phosphorylation in NIH3T3CCK-BR and Mia PaCa-2 cells. Lanes 1+4: unstimulated NIH3T3CCK-BR; 2+3: Mia PaCa-2 FAK immunoprecipitates; 5: FAK control.

Lanes 1-4 and 5 were immunoblotted with phosphotyrosine and FAK antibodies respectively.

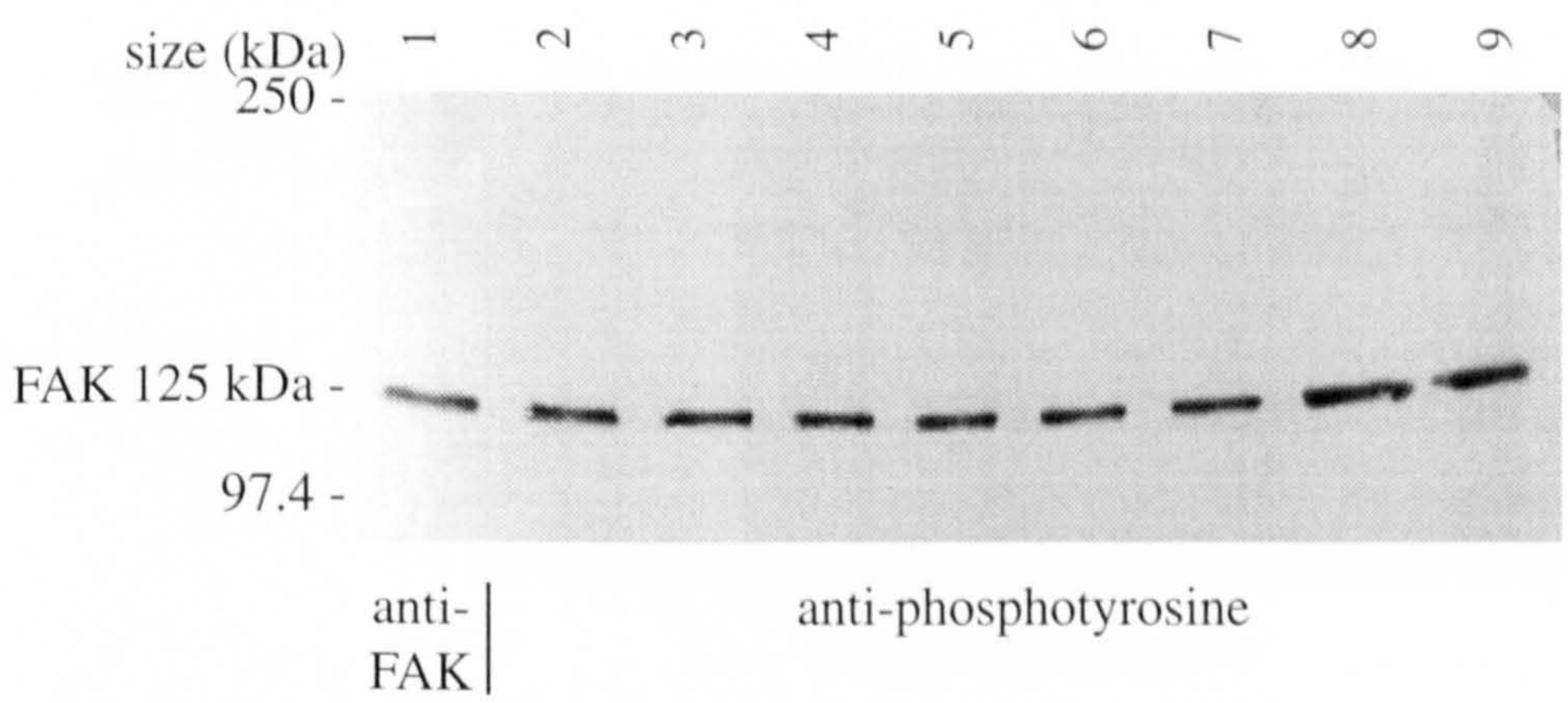


Figure 8.10 Immunoblot of 6.5% PAGE gel showing lack of effect of CCK-BR antagonists on basal FAK phosphorylation in Mia PaCa-2 cells.

Lane 1: FAK control; 2: untreated; 3: 0.1 nM RPR 102681; 4: 10 nM RPR 102681; 5: 1 μM RPR 102681; 6: untreated; 7: 0.1 nM L-740,093; 8: 10 nM L-740,093; 9: 1 μM L-740,093.

Lanes 2-9 and 1 were immunoblotted with phosphotyrosine and FAK antibodies respectively.

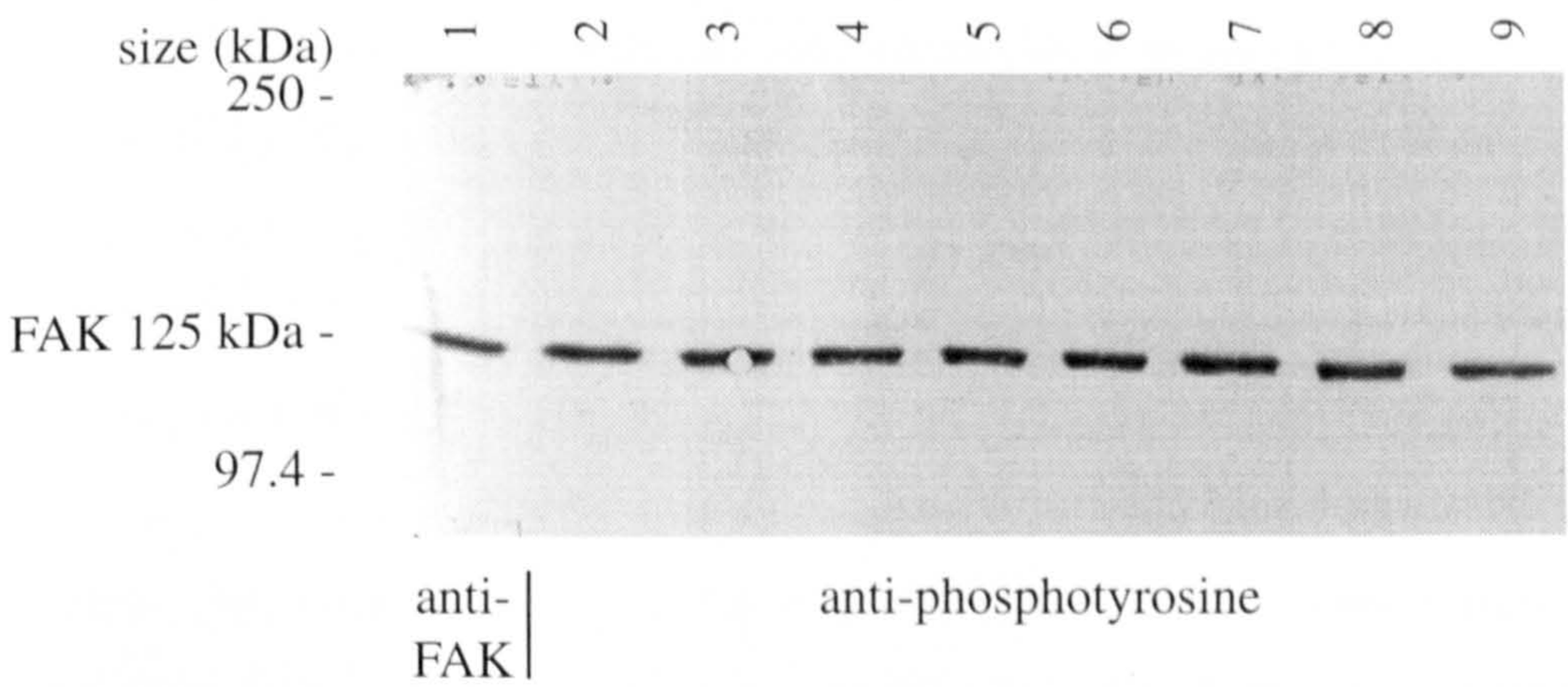


Figure 8.11 Immunoblot of 6.5% PAGE gel showing lack of effect of CCK-BR antagonists on basal FAK phosphorylation in Mia PaCa-2 cells.

Lane 1: FAK control; 2: untreated; 3: 0.1 nM L-365,260; 4: 10 nM L-365,260; 5: 1 μM L-365,260; 6: untreated; 7: 0.1 nM CI988; 8: 10 nM CI988; 9: 1 μM CI988.

Lanes 2-9 and 1 were immunoblotted with phosphotyrosine and FAK antibodies respectively.

8.6 Discussion

We found that 5 out of the 7 human pancreatic tumour cell lines possessed a point mutation in exon 1 at codon 12 of the K-*ras* gene. These results are supported by the work of O' Hara *et al.* (1986) and Sumi *et al.* (1994), who also showed that 2 of these cell lines, Panc-1 and Mia PaCa-2 had point mutations in codon 12 resulting in a GGT (glycine) to GAT (aspartate) and GTT (valine) substitution, respectively. Mutations were not found in exon 2 at codon 61 in any of the cell lines investigated. This result was not surprising, since 90% of K-*ras* mutations in human pancreatic cancer studied are located in exon 1 at codon 12 (Almoguera *et al.*, 1988; Smit *et al.*, 1988). Point mutation mediated-activation of the K-*ras* oncogene *in vivo* is restricted to codons 12, 13 and 61. These produce amino acid substitutions that reduce the GTPase activity of the encoded proteins (Walter *et al.*, 1980) resulting in an activated p21ras protein. In this form, ras continuously transmits signals to its downstream effectors such as MAPK (Marshall 1994). However, as discussed in the introduction section, the alteration in the ras protein alone is not sufficient for full malignant transformation of human pancreatic cells (Lemoine *et al.*, 1992a).

Tyrosine phosphorylation has been implicated in the intracellular signalling of neuropeptides that act as potent growth factors through receptors with seven transmembrane helices, such as bombesin, vasopressin, endothelin and bradykinin (Leeb-Lundberg & Song, 1993; Zachary *et al.*, 1992). In 1992, Zachary and co-workers showed that bombesin, vasopressin and endothelin stimulated the rapid tyrosine phosphorylation of FAK. FAK was originally found as a substrate for activated variants of *src* (family of retroviral oncogenes, Schaller *et al.*, 1992). FAK is also known to be stimulated by the integrin family of cell surface receptors (Kornberg *et al.*, 1992; Schaller *et al.*, 1992) which co-localises with several components of cellular focal adhesions, such as tensin, vinuculin and talin. Hence, it is termed focal adhesion kinase. FAK is a protein tyrosine kinase that displays unique features: for example, it does not contain SH2 or SH3 domains or acylation sites important in protein-protein interactions. The catalytic domain is located centrally between the non-catalytic amino and carboxyl termini. FAK is capable of autophosphorylation at one

site, tyrosine-397. It has been proposed by a number of researchers that FAK phosphorylation is a ras-independent mechanism (Secki *et al.*, 1995; Chen *et al.*, 1996) which was demonstrated by using dominant negative inhibitors of ras in NIH3T3 and Swiss 3T3 cells.

The work carried out on the CCK-BR signalling pathways has shown that several protein species including MAPK and FAK (Taniguchi *et al.*, 1994; Seufferlein *et al.*, 1995) are activated by phosphorylation. These findings suggest that CCK-B/gastrin receptors may transmit mitogenic signals by cross-talking with the kinase cascades. Recently, Seva *et al.* (1996) have also shown that stimulation of the gastrin/CCK-B receptor, promotes MAPK activation in a ras-dependent manner. Since ras, a potential oncogene is known to be constitutively active in 90% of pancreatic tumours, the MAPK cascade could be constitutively activated in the majority of pancreatic carcinomas. This can lead to cellular responses such as growth and differentiation which are independent of CCK receptor activation. Moreover we confirmed that 5 of these pancreatic cancer cell lines possessed a point mutation in codon 12 of the K-*ras* gene (Mia PaCa-2, Capan-1, KPan, Panc-1 and AsPc-1), theoretically removing the need for CCK receptor activation.

NIH3T3CCK-BR cells responded to sCCK-8 and nsG-17 by an elevation in FAK tyrosine phosphorylation. This was originally shown by Taniguchi *et al*. (1994) using CCK-8. The Mia PaCa-2 unstimulated cells already had phosphorylated FAK and there was no further phosphorylation by either sCCK-8 or nsG-17. The absence of further phosphorylation is also in favour of lack of sufficient CCK receptors in these cells as shown by RNP assay and radioligand binding studies. Scott & Liang (1995) have shown that an adherent human metastatic melanoma cell line (SKMEL28) expressed high levels of basal phosphorylated FAK compared with a non-adherent human metastatic melanoma line (SKMEL1) and normal human melanocytes. Moreover, FAK phosphorylation was induced by β1 integrin-activating antibodies in the normal melanocytes but not in the metastatic melanoma cells. They found that the non-adherent metastatic cells had less β1 integrin receptors than the normal melanocytes and adherent metastatic cells, thus resulting in a lack of FAK phosphorylation. The increased basal

level of phosphorylated FAK in Mia PaCa-2 cells was not reduced by the various CCK-R antagonists, indicating that this elevation is not related to activated CCK-R in these cancer cells, providing the receptor is not mutated and no longer recognised by the antagonists.

It is not surprising that basal FAK phosphorylation is higher in the cancer cells than the NIH3T3CCK-BR cells, since FAK is a point of convergence in the action of integrins, oncogenic forms of $pp60^{src}$, mitogenic neuropeptides and growth factors. In pancreatic cancer many growth factors, growth factor receptors and oncogenes are activated, which may lead to an elevated basal FAK phosphorylation status.

It may be possible that the CCK-R is mutated resulting in a constitutively active receptor. This would by-pass the effects of the CCK-R antagonists. However, this has not been reported in relation to CCK-R and cancer growth. One would have to sequence the whole of the CCK-R to determine this possibility. Another possibility is a mutation in the G-protein α subunit that is coupled to the CCK-R resulting in a constitutively activated receptor. Again, this active form of the receptor would by-pass the effects of the antagonists. Gain of function mutations in G-protein α subunits have been well documented (Lyons *et al.*, 1990; Gupta *et al.*, 1992b) for seven transmembrane GPCR in thyroid tumours. Whether the elevation in basal FAK phosphorylation is due to constitutively active CCK-R or other growth factor signalling pathways remains to be answered.

CHAPTER 9

CHARACTERISATION OF A HUMAN PANCREATIC CANCER *IN VIVO* AND *IN VITRO*

CHAPTER 9

9.1 Background

Cholecystokinin receptor studies on primary human pancreatic cancer are few and far between. However, molecular as well as protein binding studies have been carried out on animal models of pancreatic cancer. The expression of CCK-R mRNA has been examined in pancreatic carcinomas from transgenic mice and also in several rat pancreatic cancer models (Zhou *et al.*, 1993; Povoski et al., 1994). The animal models of pancreatic cancer cannot be compared directly with human pancreatic cancer due to variations in morphology and genetics. RT-PCR demonstrated the expression of both CCK-A and CCK-B receptors in the normal human pancreas (Zhou *et al.*, 1994). However,one of the questions addressed here is whether there is a differential expression of the CCK-A and CCK-B receptors in primary pancreatic cancers compared with their matched normal specimens.

9.2 Aims

Attempts were made to a) xenograft, b) passage tumours c) develop a stable cell line, from more than one primary human pancreatic cancer but all three aims were successful in only one case, PT1.

The aims of these investigations were; i) to compare the CCK-R and K-ras status on the xenograft established *in vivo* and the cell line cultured *in vitro* from a primary human pancreatic tumour 1 (PT1); ii) to determine the differential expression of CCK-A and CCK-B receptors in 4 human pancreatic cancers with their matched 'normal' counterparts by RT-PCR (PTN1-PTN4). Two sets of patient pancreatic specimens were collected for these studies; a) pancreatic tumour tissue without matched normal specimens which were used for the *in vivo* and *in vitro* studies and b) pancreatic tumours with matched 'normal' specimens (from the same patient) which were used for the CCK-R differential expression studies.

9.3 Molecular studies to determine CCK-R status in a human pancreatic cancer and its xenografts

9.3.1 *Methods*

9.3.1.1 *CCK-R RT-PCR*

The methods are described in section 4.1.3-4.1.7. Total RNA was extracted from the primary tumour (PT1), xenografts of this tumour and the cell line established from this tumour. The RNA was reverse-transcribed into cDNA and the cDNA was amplified using the CCK-R specific primers by PCR. The amplified products were then subjected to agarose gel electrophoresis.

9.3.2 *Results*

Tumour PT1 was obtained from a 56 yr. old female patient who was diagnosed with stage IV pancreatic cancer. PT1 was 1 of 5 other pancreatic tumours that grew *in vivo* as well as *in vitro*. A cell line was established from the xenograft of this tumour in passage 7. Attempts at growing the tumour *in vitro* from the original tumour and from xenografts of the first and second passage were unsuccessful.

Pancreatic tumour	Sex	Age	Stage of cancer
PT1	female	56	IV, T3, N1, M1
PT2	female	46	III, T2, N1, M0
PT3	male	58	II, T3, N0, M0
PT4	male	59	I, T1, N0, M0
PT5	female	43	III, T3, N1, M0

Table 9.1 Details of patients and tumour specimens analysed for *in vivo* and *in vitro* pancreatic tumour growth establishment. T: tumour extension; N: lymph node involvement; M: metastasis.

RT-PCR showed the expression of both CCK-A and CCK-B/gastrin receptors in the original pancreatic tumour (PT1), the xenografted tumours (passages 2, 5 and 7) and in the cell line (established from tumour xenograft at passage 7 from PT1) as shown in Figure 9.1. The T cell lymphoma cell line was used as a positive control for both CCK-AR and CCK-BR (lanes 6A and 6B) and NIH3T3 mouse fibroblast cells were used as a negative control as indicated in lanes 7A and 7B in Figure 9.1. The results obtained for the CCK-A and CCK-B/gastrin receptor PCR in the NIH3T3CCK-BR cells are shown in Figure 9.2. Lane 2A shows a lack of expression for the CCK-AR whereas lane 2B shows the expression of the human CCK-BR in the NIH3T3CCK-BR cells. The last three lanes verify the presence of intact cDNA in the NIH3T3 and NIH3T3CCK-BR cells by the amplification of a 120 bp β-actin fragment. There was not enough tumour material available for CCK-R expression analysis with tumours PT2-PT5 as they were used for *in vivo* and *in vitro* studies.

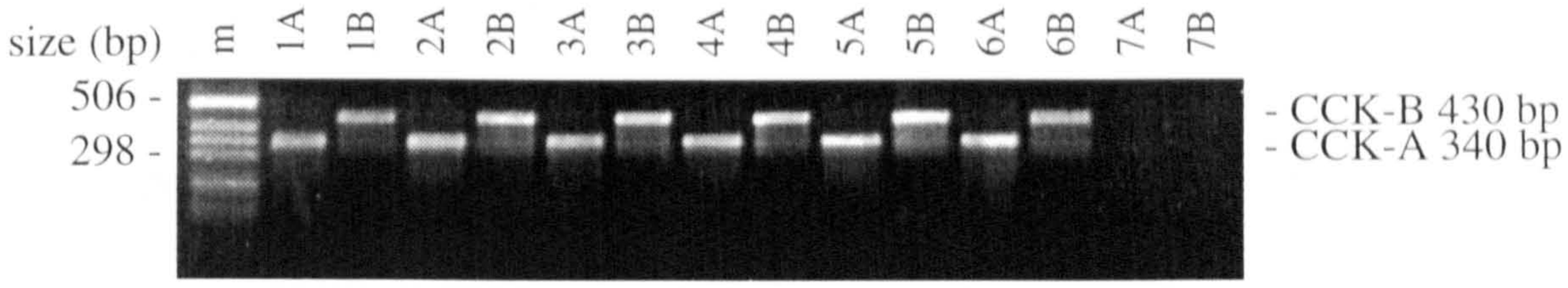


Figure 9.1 Agarose gel electrophoresis of CCK-AR and CCK-BR RT-PCR products in a human pancreatic tumour, PT1.

The size of the amplified fragments were 340bp and 430bp for the CCK-AR and CCK-BR respectively. Expression studies were carried out on the original human pancreatic tumour (PT1) and on the subsequent PT1 xenografts from mice and PT1 cell line cultured *in vitro*. A T-cell line and NIH3T3 cells were used as a positive and negative control respectively.

Lanes 1A,1B: primary tumour; 2A,2B: tumour cell line; 3A,3B: xenograft from passage 2; 4A,4B: xenograft from passage 5; 5A,5B: xenograft from passage 7; 6A,6B: T cell line; 7A,7B: NIH3T3 cells; m: molecular markers.

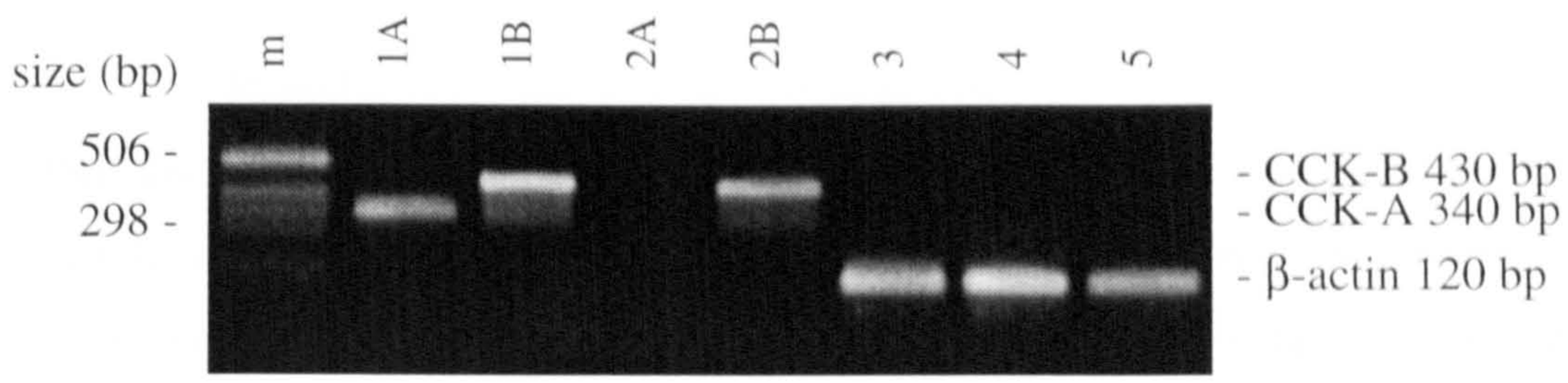


Figure 9.2 Agarose gel electrophoresis of CCK-AR and CCK-BR RT-PCR products in NIH3T3 and NIH3T3CCK-BR cells.

The size of the amplified fragments were 340bp and 430bp for the CCK-AR and CCK-BR respectively.

Lanes 1A, 1B: T cell line (positive control); 2A, 2B: NIH3T3CCK-BR cells; 3-5 represent the β-actin gene expression. Lane 3: wild type NIH3T3 cells; 4: NIH3T3CCK-BR cells; 5: T cell line; m: molecular markers.

9.3.3 *Methods*

9.3.3.1 *RNase protection assay*

The methods are described in section 4.4. Total RNA from the primary pancreatic cancer and its xenografts was hybridised with the radioactively labelled CCK-AR or CCK-BR probe overnight. The unhybridised RNA (single stranded) was digested with RNase and the remaining product electrophoresed on a polyacrylamide gel. Kodak film was exposed to the gel for several days before developing.

9.3.4 *Results*

The RNP assays did not detect expression of either the CCK-A or CCK-B/gastrin receptors in the pancreatic cancer samples analysed from PT1 and its subsequent xenografts and cell line as shown in Figures 9.3 and 9.4 respectively. Human gall bladder revealed a 340 bp protected CCK-AR mRNA and the NIH3T3CCK-BR cells revealed a clear band of the expected size for the protected CCK-B/gastrin (430 bp) receptor mRNA

Figure 9.3 Autoradiography of 6% PAGE gel following the RNP assay. The CCK-AR mRNA was not detected in the human pancreatic cancer (PT1) but was present in human gall bladder (lane 1).

Expression studies were carried out on the original human pancreatic tumour (PT1) and on the subsequent PT1 xenografts from mice and the PT1 cell line cultured *in vitro*. Human gall bladder and Mia PaCa-2 cells were used as positive and negative controls respectively.

Lane 1: human gall bladder (positive control); 2: original pancreatic tumour; 3: tumour cell line; 4 xenografts: passage 2; 5: passage 5; 6: passage 7; 7: NIH3T3 cells; 8: Mia PaCa-2 (negative control); m: molecular markers.

The protected fragments were resolved on a 6% polyacrylamide gel at 55W for 5 hrs in 1 x TBE buffer.

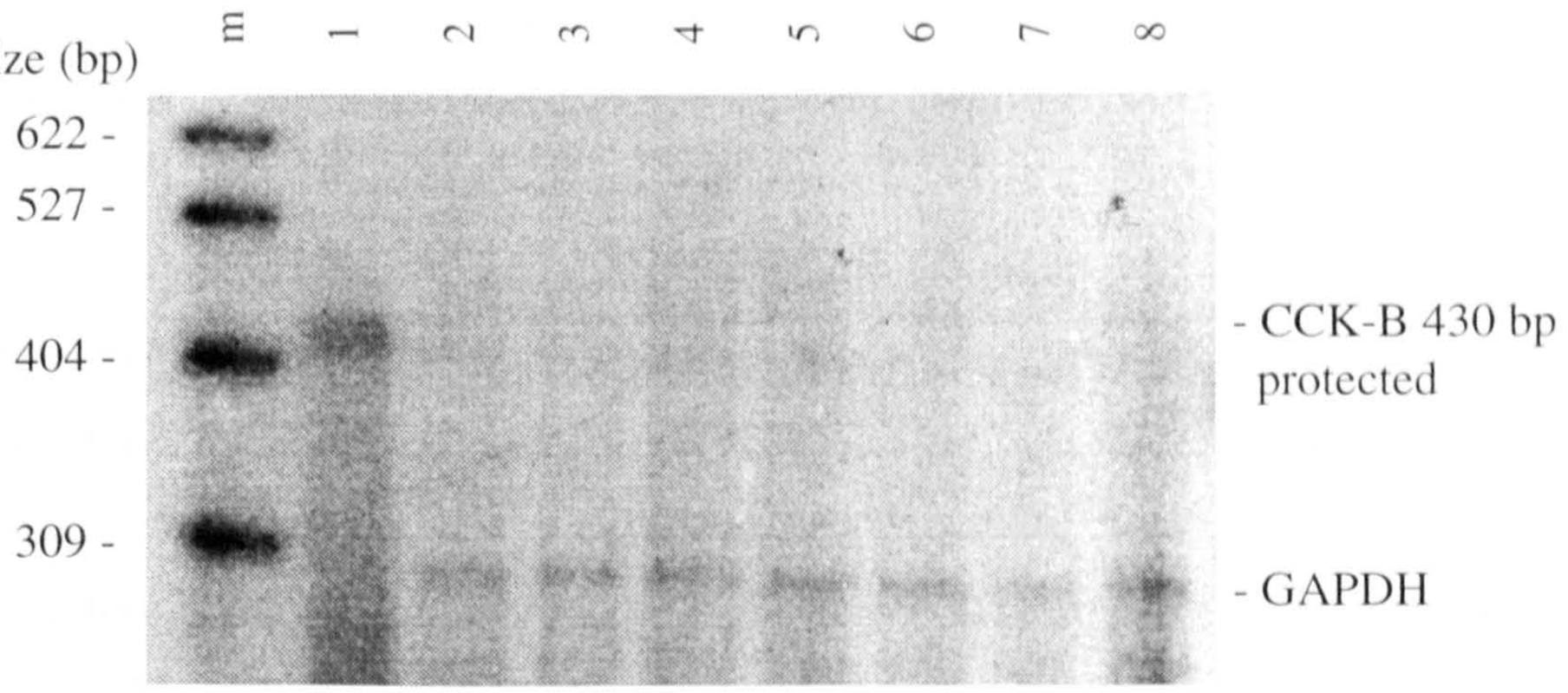


Figure 9.4 Autoradiography of 6% PAGE gel following the RNP assay. The CCK-BR mRNA was not detected in the human pancreatic cancer (PT1) but was present in NIH3T3CCK-BR cells (lane 1).

Expression studies were carried out on the original human pancreatic tumour (PT1) and on the subsequent PT1 xenografts from mice and the PT1 cell line cultured *in vitro*. NIH3T3CCK-BR and Mia PaCa-2 cells were used as positive and negative controls respectively.

Lane 1: NIH3T3CCK-BR (positive control); 2: original pancreatic tumour; 3: tumour cell line; 4 xenografts: passage 2; 5: passage 5; 6: passage 7; 7: NIH3T3 cells; 8: Mia PaCa-2 (negative control); m: molecular markers.

The protected fragments were resolved on a 6% polyacrylamide gel at 55W for 5 hrs in 1 x TBE buffer.

9.4 The K-*ras* status in the human pancreatic cancer, PT1

9.4.1 *Methods*

The methods are described in section 4.6.2. K-*ras* exon 1 and 2 were amplified, cloned and sequenced. The sequenced products were subjected to polyacrylamide gel electrophoresis. Kodak film was exposed to the gel overnight before developing.

9.4.2 Results

Cloning and sequencing of the K-*ras* PCR products showed that the original PT1 tumour and its first passage xenograft possessed a wild type *ras* sequence. However, there was a mutation in codon 12 of exon 1 from the second passage onwards of PT1. The cell line established from passage 7 of this tumour also had a point mutation in codon 12. The substitution was from the wild type glycine to a valine at codon 12 as indicated by a base point mutation from guanine (G) to a thymine (T) in Figure 9.5. There was no mutation detected in the hot spot region of codon 61 in exon 2 in the pancreatic cancer specimen or the xenograft passages investigated as shown in Figure 9.6. The mutations detected are summarised in Table 9.2.

Human pancreatic tumour 1 (PT1)	K-*ras* Exon 1 codon 12	Exon 2 codon 61
original tumour	GGT	CAA
passage one	GGT	CAA
passage two*	G<u>T</u>T	CAA
passage three*	G<u>T</u>T	CAA
passage five*	G<u>T</u>T	CAA
passage seven*	G<u>T</u>T	CAA
tumour cell line*	G<u>T</u>T	CAA

Table 9.2 K-*ras* mutations in the xenografted human pancreatic cancer, PT1 and its cell line (passage 7). The point mutations* in exon 1 at codon 12 are underlined. GGT: glycine, GTT: valine, CAA: glutamine. No mutations were found at codon 61.

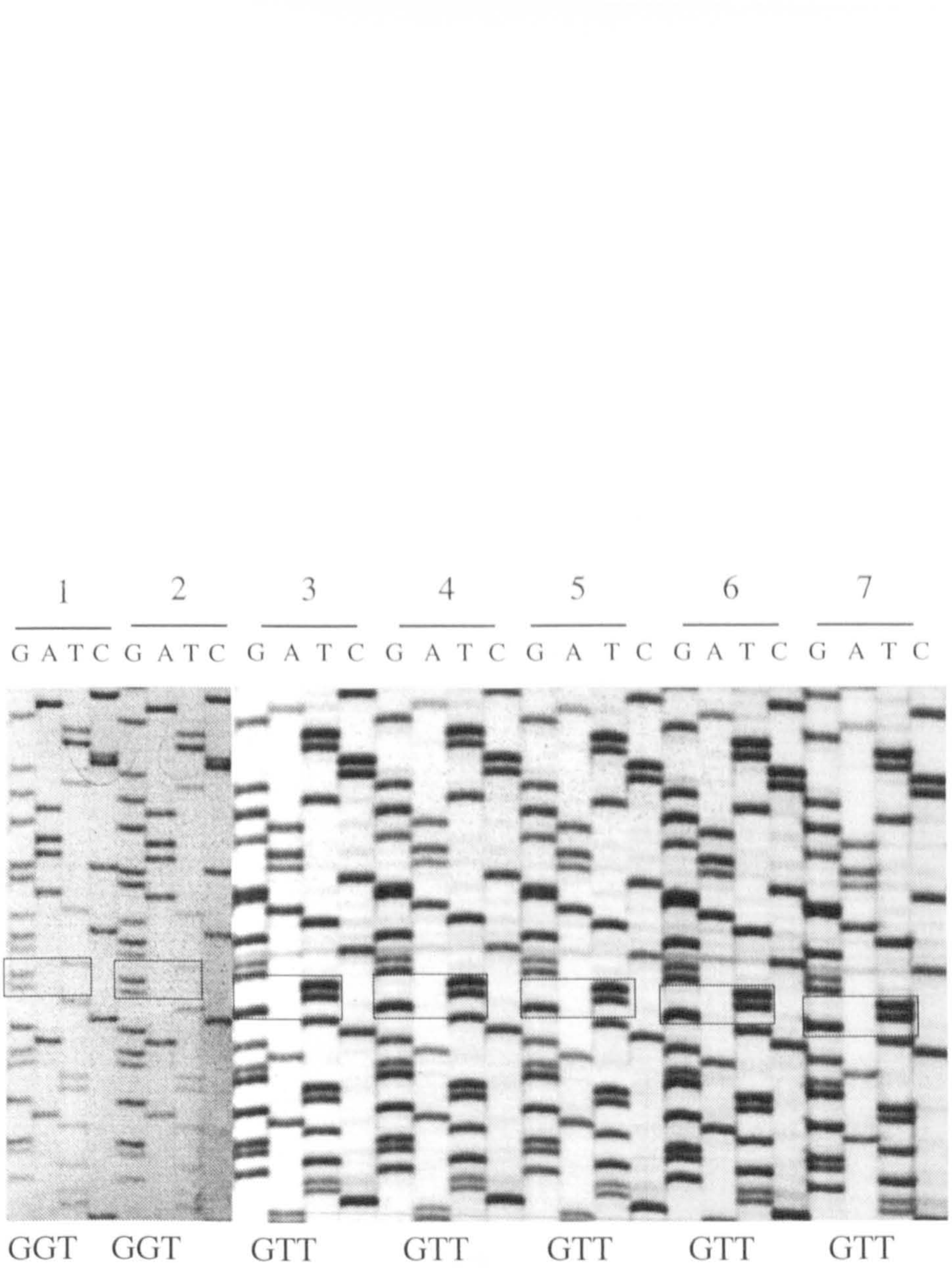


Figure 9.5 Sequences of the K-*ras* gene exon 1 in codon region 11-18 in a human pancreatic cancer (PT1) and subsequent xenografts and PT1 cell line. Mutation from wild type GGT (glycine: left lanes) to GTT (valine: right lanes) was detected in codon 12.

Lane 1: original tumour; lane 2: xenografts from first passage; 3: second passage; 4: third passage; 5: fourth passage; 6: seventh passage; 7: cell line established from tumour passage 7. The boxes enclose the codon 12 triplet.

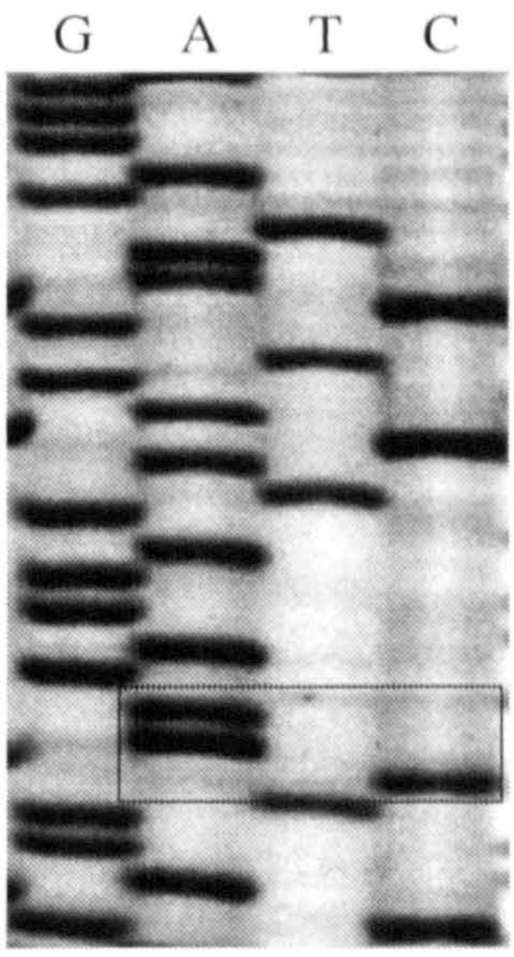


Figure 9.6 Sequences of the K-*ras* gene exon 2 in codon region 58-64 in a human pancreatic cancer (PT1) and subsequent xenografts and PT1 cell line. Mutations were undetected. The sequence of the codon 61 wild type CAA (glutamine) is enclosed in the box.

Table 9.3 shows that as the passage number of the xenograft increased, the time for the tumour to form decreased. This tumour was the only tumour that was successfully passaged from a total of 5 human pancreatic cancers that were xenografted.

Human pancreatic tumour passage	**Time (days)**	**sem**
One	76	4.00
Two	60	7.30
Three	35	6.30
Four	22	2.00
Five	20	2.25
Six	12	3.00
Seven	10	3.00

Table 9.3 The human pancreatic cancer PT1 xenograft in nude mice: Tumour growth time decreases as the number of passages increase.

9.5 Semi-quantitative CCK-R RT-PCR in human pancreatic cancer with matched normal tissues

9.5.1 *Methods*

The methods are described in section 4.7. The cDNA from pancreatic tumours and matched normal tissue was diluted and amplified using the CCK-R specific primers by PCR in the presence of β-actin. The amplified products were then subjected to agarose gel electrophoresis.

9.5.2 *Results*

Semi-quantitative RT-PCR was carried out on 4 human pancreatic cancers with their matched normal tissues (PTN1, PTN2, PTN3 and PTN4). In Figure 9.7 all 4 normal and tumour specimens showed the expression of both CCK-A and CCK-B receptors. As the cDNA was diluted down to 1:10,000 the message for both CCK-AR and CCK-BR was lost in both cancers and 'normal' tissues. All the pancreatic cancer specimens along with their matched 'normals' show that both CCK-A and CCK-B receptors were expressed. These results indicate that the CCK-R were equally expressed in the matched 'normal' and tumour specimens, as the message for these receptors was lost at the same dilutions in both the cancer and normal pancreatic tissues.

Pancreatic tumour with matched normal	Sex	Age	Stage of cancer
PTN1	male	57	I, T1, N0, M0
PTN2	male	68	I, T1, N0, M0
PTN3	male	65	I, T1, N0, M0
PTN4	female	63	I, T1, N0, M0

Table 9.4 Details of patients and tumour specimens with matched normal pancreatic tissue analysed for semiquantitative CCK-R RT-PCR expression studies. T: tumour extension; N: lymph node involvement; M: metastasis.

Figures 9.7A, B and D may indicate higher expression of CCK-AR than CCK-BR in the tumour samples although this is not reflected in Figure 9.7C.

However RT-PCR is not a quantitative method in order to make this assumption when comparing PCR products of differing lengths (340 and 430 bp for CCK-AR and CCK-BR respectively). Table 9.4 shows the tumour and patient details of each specimen analysed in this study.

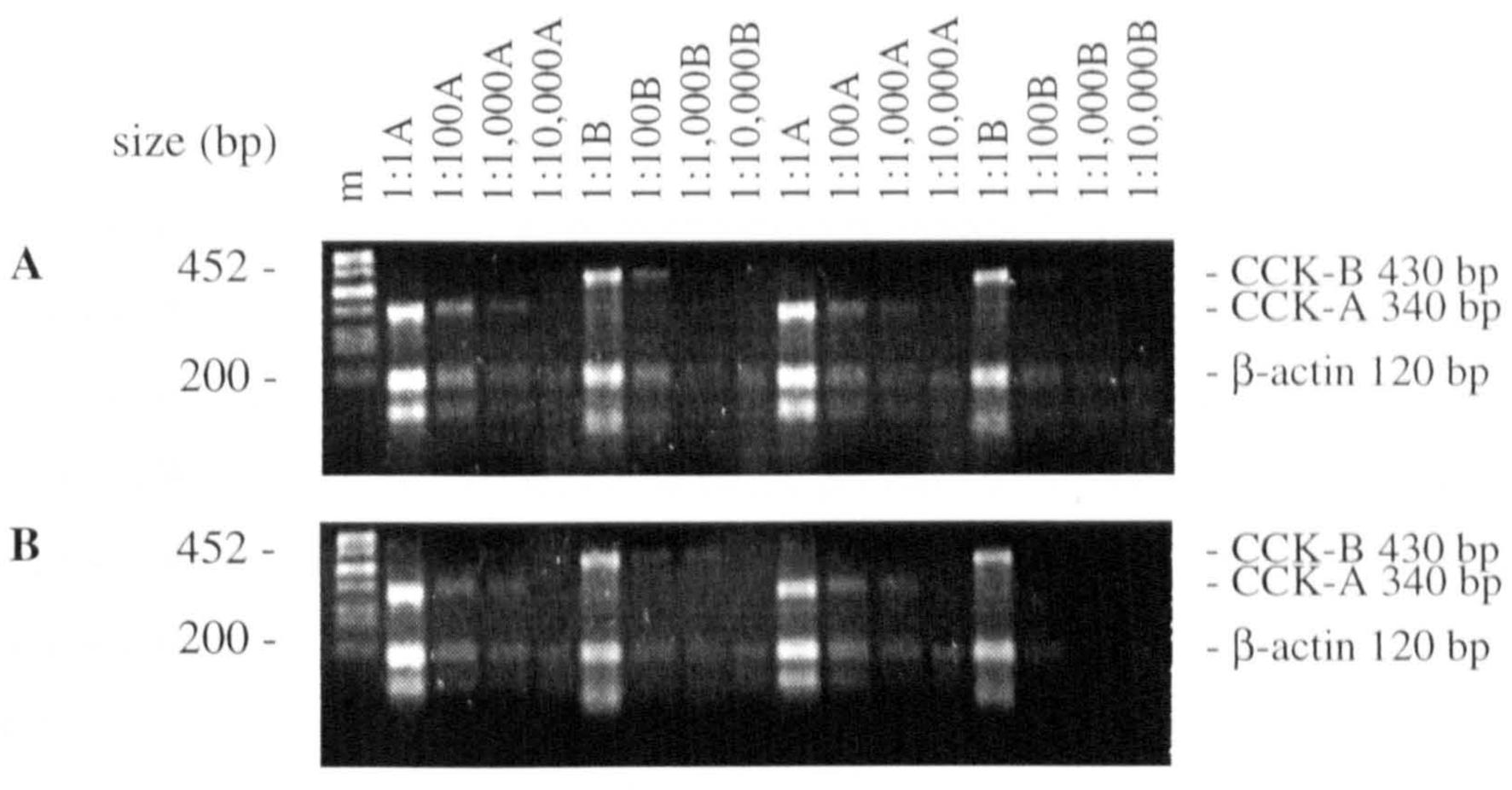


Figure 9.7 Agarose gel electrophoresis showing semi-quantitative CCK-A and CCK-B receptor RT-PCR in four human pancreatic cancers with matched normal tissue. A to D are pancreatic tumour specimens with matched normals, PTN1 (A), PTN2 (B), PTN3 (C) and PTN4 (D).

9.6 Discussion

The RT-PCR studies show that both CCK-A and CCK-B receptors are expressed in the human pancreatic cancer PT1, but the receptors were not detected utilising the RNP assay which had a detection limit of 10 pg for CCK receptor mRNA. As mentioned previously, the CCK-A and CCK-B receptors were detected by RNP in the human gall bladder and NIH3T3CCK-BR cells, respectively. In these two controls the receptors are known to have a role in either muscle contraction (gall bladder) or cell growth (NIH3T3CCK-BR). Thus, these results in the primary pancreatic cancer indicate that the receptor message is expressed at very low levels at which they are probably unable to elicit a response. The cell line established from the tumour also lacked significant CCK-R expression as shown by RNP assays (Figures 9.3 and 9.4).

Freshly isolated cultures from an original tumour are known as primary cultures until they are passaged or subcultured. They are usually heterogeneous and have a low growth rate but are more representative of the cell types in the tissue from which they were derived. Subculture allows the expansion of a culture (known as a cell line). After several subcultures a cell line will either die out (finite cell line) or 'transform' to become a continuous cell line.

We have shown the gain of a K-*ras* mutation from tumour passage 2. The original tumour and the primary passaged tumour displayed a wild type K-*ras* gene. This finding is not surprising as it is well documented that upon tumour passaging whether *in vivo* or *in vitro,* the faster growing cells are selected, leading to the survival of the fittest clones (Nowell, 1976). Generally these faster growing cells gain various genetic mutations that confer a growth advantage such as a ras mutation, hence outcompeting the slower dividing cells. This also explains the reduction in time from which the tumour was injected to the time when it was palpable with an increase in passage number, as well due to the involvement of other oncogenic mutations such as p53. The original and primary tumour was difficult to grow *in vitro*. However, the xenografted tumour taken from passage 7 resulted in cells growing *in vitro* supplemented with 15% foetal calf serum. However, these cells could not be grown in serum-free medium.

The cells were weaned on medium containing varying amounts of foetal calf serum. The serum content was reduced from 15%, to 10, 5, 2.5 and 0% foetal calf serum with time. At 5% foetal calf serum the cells began to die. This can be due to the cells being partially transformed (*eg.* ras mutation) but not yet able to grow independent of growth factors such as epidermal growth factor and insulin that are provided by the serum. Continuous cell lines have an advantage of an increase in growth rate to higher cell densities, their lower serum requirement and general ease of maintenance in simple media. However, their disadvantages include greater chromosomal instability, divergence from the donor phenotype and loss of tissue specific markers.

We were unable to establish any other fresh pancreatic cancer obtained from theatre as a xenograft in nude mice or as a cell line. PT1 was the only carcinoma that was successfully passaged out of 5 human pancreatic cancers. The same procedure was carried out with all the 4 tumours in that they were injected into nude mice within 60 min of receiving the fresh specimen from theatre ensuring that the tumours did not dry out and aseptic techniques were used whilst handling the tumours. One explanation for the lack of tumour growth in these mice may be the stage of the disease when the tumour was resected. The details of the specimens analysed are shown in Table 9.1. Interestingly the PT1 specimen was taken at a more advanced stage of pancreatic cancer (stage IV). At this stage the tumour had spread to the lymph nodes as well as other organs indicating a more aggressive cancer than stage II and III cancers used. The other specimens used for this study were stage II or III tumours and this may reflect crucial properties of the tumour in order to establish a xenograft in the nude mice as well as a cell line *in vitro.*

Pancreatic tumours with matched normal tissues were difficult to obtain. However, all the tumours investigated indicate equal expression of both CCK-A and CCK-B receptors by RT-PCR compared with their matched normal specimens of pancreas. Semi-quantitative RT-PCR is a method that can be used when the source is a limiting factor and is sensitive enough to detect a single molecule of mRNA. Other methods such as RNP assays and Northern blotting can be used when the source of RNA is abundant. The RNP assay was not used in this study due to the lack of sufficient

pancreatic tissue. The tumours investigated in the RT-PCR study were classified T1, N0, M0 indicating that there was no lymph node involvement or metastasis to other organs. Whether there would be increase in CCK-R expression at a later stage of the disease remains to be determined. However it would be difficult to obtain matched normal pancreatic tissue at more advanced stages in order to make comparisons since these are rarely operated on.

This is the first study to my knowledge on CCK-R expression in human pancreatic cancers with matched normal controls. Expression studies carried out in azaserine-induced rat pancreatic cancers have shown over-expression of the CCK-AR (Bell *et al.*, 1992) and novel expression of the CCK-BR (Zhou *et al.*, 1992) mRNA. This was shown by CCK-8 binding and Northern blot analysis respectively. The binding of [^{125}I]gastrin-I was detected only in the precursor lesions of pancreatic cancer and in the cancer itself (Povoski *et al.*, 1993b). Later, Povoski *et al.* (1994) showed by RT-PCR that CCK-AR expression was similar in transgenic mouse pancreatic cancers and normal nontransgenic mouse pancreas as we have found in human pancreatic specimens.

The data obtained from human pancreatic carcinomas cannot be compared directly to animal models as morphological and genetic evidence indicates that the cellular origin of malignant lesions differs in rats, hamsters and humans. For example, activation of the K-*ras* gene is frequent in human pancreatic cancer and in *N*-nitrosobis (2-oxopropyl) amine (BOP)-induced pancreatic cancer in the hamster, but is not found in azaserine-induced lesions in rats (Bos, 1989; Van Kranen, *et al.*, 1991). Also carcinogens in rats induce lesions of acinar cell origin, a form of malignancy that is less common in humans (Longnecker, 1986; McGuiness *et al.*, 1987) as 90% of human pancreatic tumours are of ductal origin (Morohoshi *et al.*, 1983).

However, RT-PCR is exquisitely sensitive and may be detecting a very low copy number of mRNA molecules. Furthermore, demonstration of receptor mRNA does not

always reflect the presence of functional receptors in the cell membrane. Due to the paucity of primary tissue it was impossible to carry out any form of receptor protein expression studies, such as radioligand binding analysis, to indicate the presence of the receptor protein. This may be a general problem with human pancreatic cancer, resulting in a lack of research in this field.

CHAPTER 10

IN VIVO XENOGRAFT STUDIES

CHAPTER 10

10.1 Background

The transplantation of human tumour cells into immunodeficient mice has provided an *in vivo* model for the study of biological behaviour and tumour progression. Although extrapolation from animal to man is difficult, working directly on human tumours has the advantage that it is possible to focus the studies on particular types of cancer. This model allows the effects of various external factors on human tumour growth to be studied *in vivo* in an environment that is more physiological compared to *in vitro* cell culture studies.

10.2 Aims

The aims of this study were (1) to establish pancreatic cancer cell line xenografts in immunodeficient mice (2) to investigate the effect of gastrointestinal hormones, sCCK-8 and nsG-17 on the growth of human pancreatic cancer cell line (Mia PaCa-2) xenografts (3) to determine the effects of novel specific CCK-R antagonists on Mia PaCa-2 xenograft growth.

10.3 Establishment of human pancreatic cancer cell line xenografts in nude and SCID mice

10.3.1 *Methods*

The methods are described in section 5.5.1. The various pancreatic cancer cell lines were cultured *in vitro* and the harvested cells resuspended in tissue culture medium devoid of serum. These cells were injected into either SCID or nude mice maintained in sterile conditions.

The histopathological studies on the xenografts were carried out by Dr. S. Humphries using the basic haematoxylin and eosin staining technique.

10.3.2 *Results*

	IN VITRO		*IN VIVO*				
Pancreatic cancer cell line	**Cell culture Time (days, mean)**	**sem**	**SCID Time (days, mean)**	**sem**	**Nude Time (days, mean)**	**sem**	**n**
Mia PaCa-2	1.50	0.09	6.0	0	3.8	0.5	4
Panc-1	2.5	0.13	18.5	1	10.5	0.5	4
BxPc-3	2.0	0.05	11.5	0.5	6.5	0.6	4
Hs766T	3.0	0.1	31.5	1.4	23.5	0.8	4

Table 10.1 Time taken for the xenografted human pancreatic cancer cell lines to form a palpable tumour in SCID and nude mice and their doubling time *in vitro*. The tumours formed at a faster rate in the nude mice.

The time taken for the formation of a palpable tumour in two strains of mice varied with each cell line xenografted (Table 10.1). The Mia PaCa-2 cells were first to induce a tumour followed by BxPc-3, Panc-1 and Hs766T. This is also reflected by the doubling time of the cells in *in vitro* growth studies. The histopathology data on human pancreatic cancer cell line xenografts from both SCID and nude mice demonstrated identical histopathology. The Mia PaCa-2 (Figure 10.1A) and Panc-1 (Figure 10.1B) xenografts were shown to be poorly differentiated epithelial tumours. The AsPc-1 xenografts were found to be a differentiated carcinoma with glandular structures as shown in Figure 10.1C and the BxPc-3 xenografts showed a very well differentiated adenocarcinoma with mucin secreting cells (Figure 10.1D).

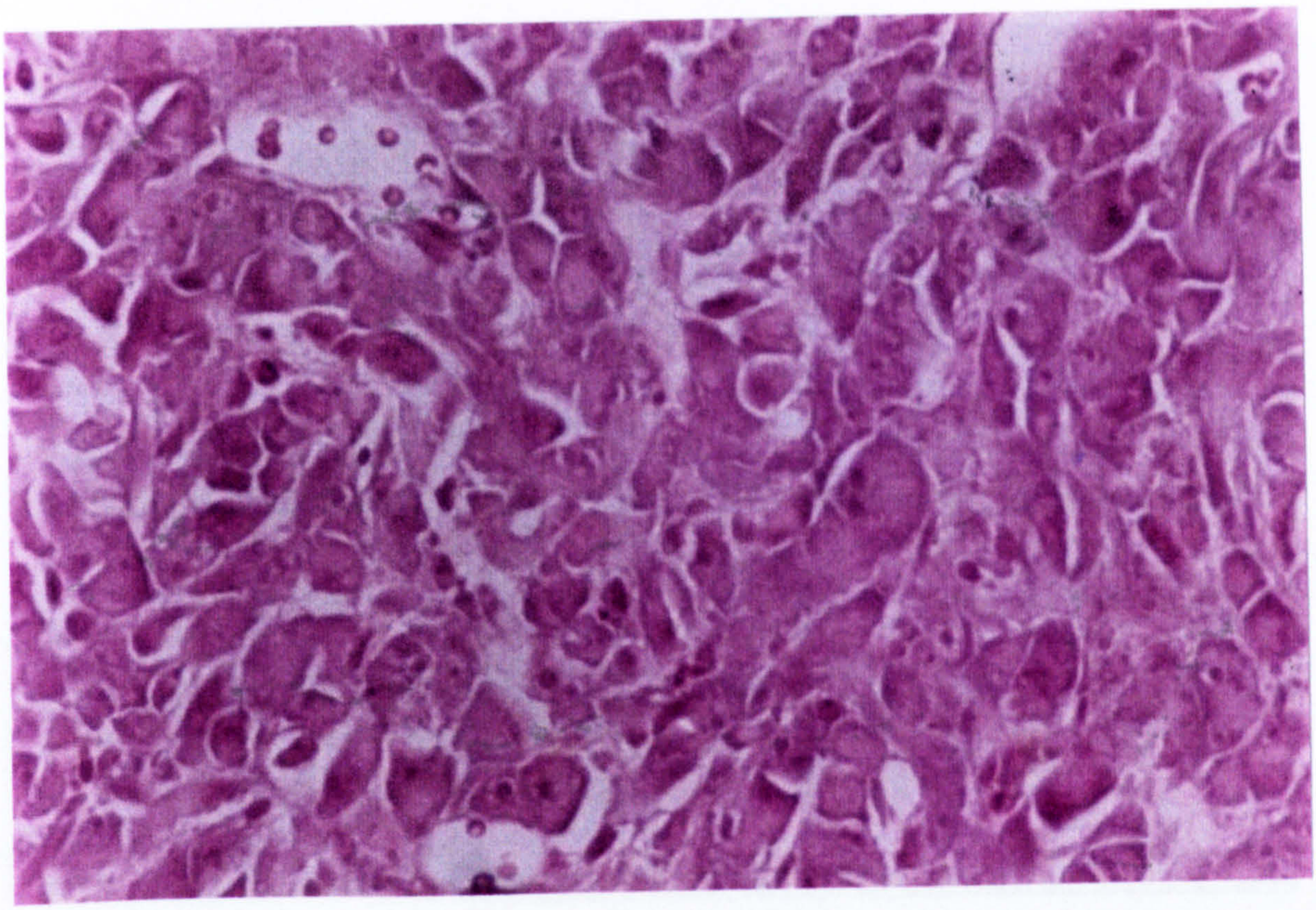


Figure 10.1A Mia PaCa-2 xenograft taken from nude mice: a poorly differentiated epithelial carcinoma with pleomorphic malignant epithelial cells. (H+E, 400X).

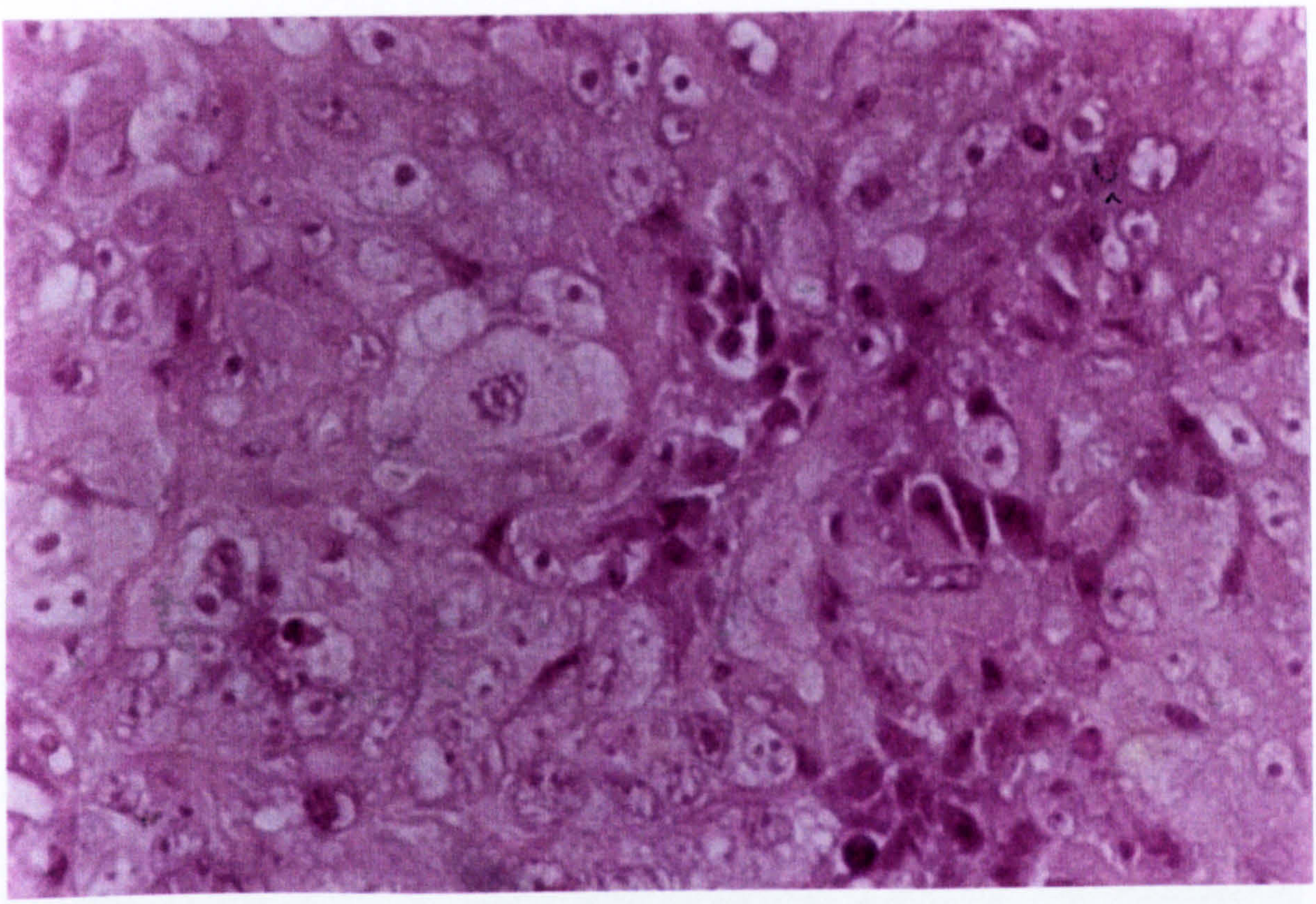


Figure 10.1B Panc-1 xenograft taken from nude mice: a poorly differentiated epithelial carcinoma with pleomorphic malignant epithelial cells (very similar in phenotype to the Mia PaCa-2 xenograft). (H+E, 400X).

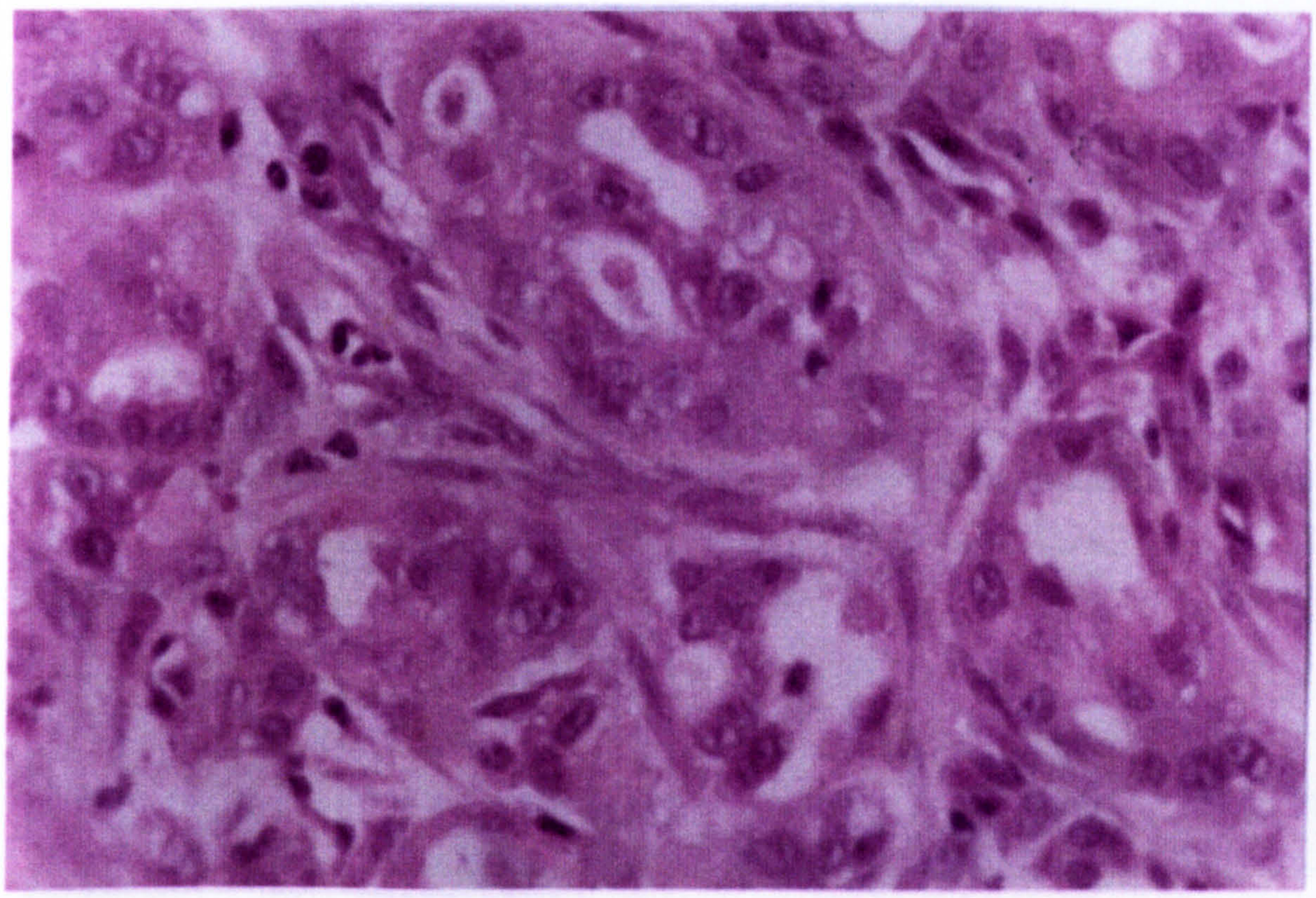


Figure 10.1C AsPc-1 xenograft taken from nude mice: a differentiated carcinoma with glandular structures. (H+E, 400X).

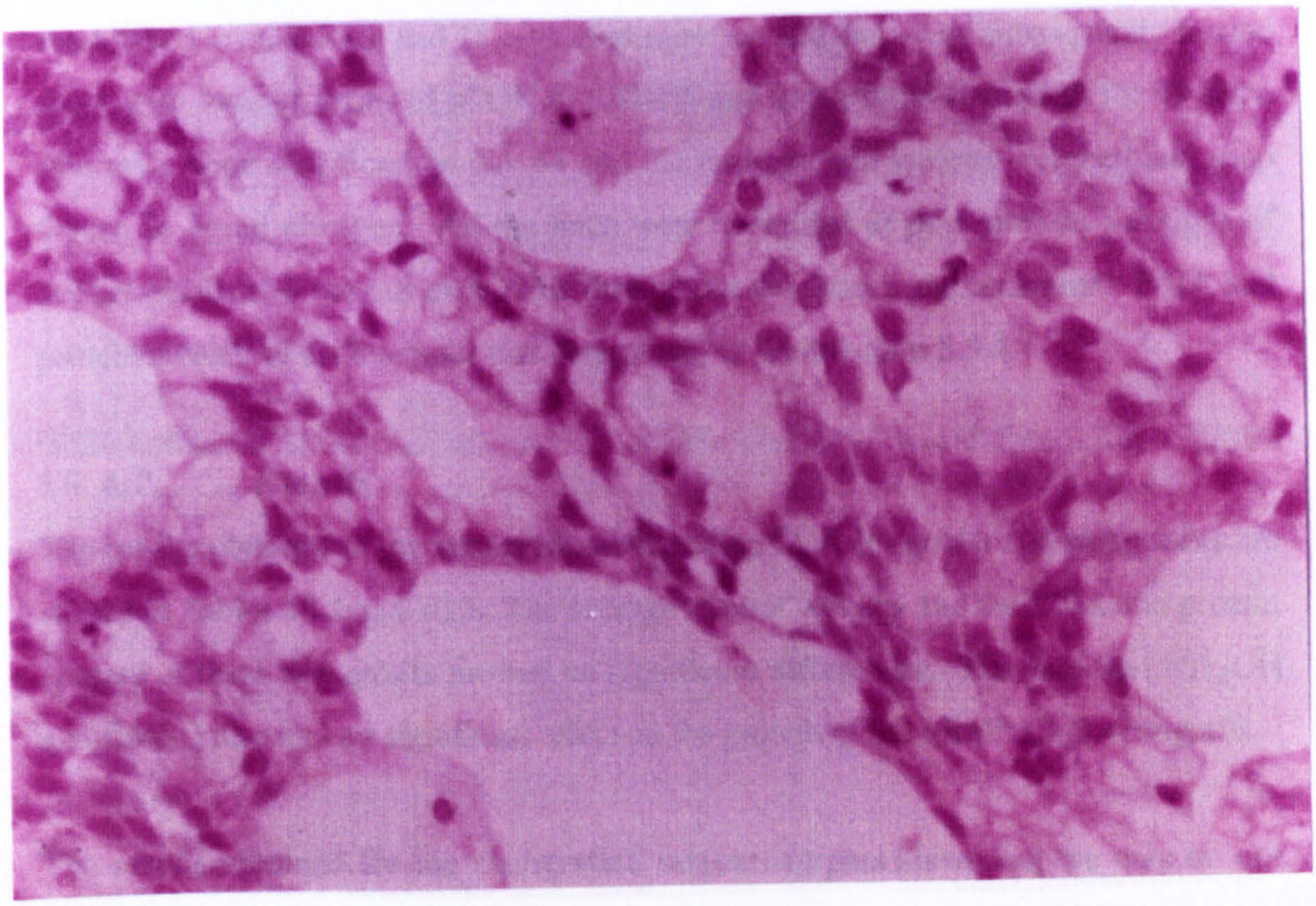


Figure 10.1D BxPc-3 xenograft taken from nude mice: a very well differentiated adenocarcinoma with mucin secreting goblet cells. (H+E, 250X).

10.4 The effects of sCCK-8 and nsG-17 on the growth of human pancreatic cancer cell line xenografts

10.4.1 *Methods*

The methods are described in section 5.5 to 5.5.4. The Mia PaCa-2 cells were established in nude mice and 15 days after injection and they were subsequently treated daily with various doses of sCCK-8, nsG-17 and antagonists (1 mg/kg) for 49 days. The mice were sacrificed on day 64 and the tumours and spleens weighed and snap frozen in liquid nitrogen. All the mice were examined at post mortem for signs of abnormalities and tumour metastasis throughout the thorax and abdomen. DNA and protein content of each tumour was determined as described in 5.5.4.

10.4.2 *Results*

The results obtained in the xenograft studies using CCK-R agonist and antagonists are shown in Tables 10.2 and 10.3. The average tumour weights and the DNA and protein content of these xenografts showed no significant differences. The vehicles, NH_4OH and DMSO also had no significant effect on the growth of the Mia PaCa-2 xenografts.

The results obtained for the xenografted tumour weights show that the data do not conform to a normal distribution (see Table 1 in appendix II, p292). If the data were distributed normally one would have expected 95% of the data to be encompassed by twice the SD value (2 x SD). However, for all treated and untreated groups the 2 x SD values are less than zero, which is not the case in a normally distributed population. Table 2 in the appendix II shows that the coefficient of variation was greater than 100% in 15 out of the 21 groups, *i.e.* the SD is more than the mean value of the group, which further implies that the data do not conform to a normal distribution. This is also confirmed by the difference between the mean and median values of the tumour weights. For normally distributed data these would typically be very close, but this is not the case (see Table 2 in appendix II, p293). When data available for analysis do not fit the normal distribution population, one can avoid a more complicated model of analysis by transforming the data such that it makes the normal distribution model appropriate. This is done by logarithmic or square root transformation of the original

data. Unfortunately, logarithms of the data cannot be taken as there are a number of zero values and the square roots still result in a skewed distribution. Hence, parametric statistical analysis cannot be used to assess significance for this data.

Treatment group	DMSO			PBS/NH_4OH		
	Tumour weight (g)			Tumour weight (g)		
	mean	sem	n	mean	sem	n
untreated	0.48	0.17	12	0.48	0.17	12
vehicle	0.31	0.09	12	0.05	0.03	6
15 μg/kg sCCK-8	0.32	0.24	6	0.46	0.17	6
30 μg/kg sCCK-8	0.53	0.25	6	0.24	0.24	5
100 μg/kg sCCK-8	0.58	0.31	6	0.52	0.23	6
15 μg/kg nsG-17	0.55	0.28	5	0.46	0.19	6
30 μg/kg nsG-17	0.25	0.06	6	0.31	0.07	6
100 μg/kg nsG-17	1.04	0.41	5	0.54	0.22	6
1 mg/kg L-364,718	0.44	0.18	9	-	-	
1 mg/kg L-365,260	0.57	0.30	9	-	-	
1 mg/kg L-740,093	0.67	0.29	9	-	-	
1 mg/kg RPR-X	0.37	0.17	8	-	-	
20 mg/kg JB93182	0.13	0.10	6	-	-	
JB93182 vehicle (saline)	0.31	0.12	6	-	-	

Table 10.2 Human pancreatic cancer xenograft weights were unaffected by sCCK-8, nsG-17 and their antagonists, although the effects of sCCK-8 and nsG-17 at higher doses are associated with higher average tumour weights (not significantly different).

The first statistical analysis of the data considered the solvents. A non-parametric Kruskal-Wallis one way analysis of variance was carried out on the following groups: untreated control, saline, DMSO and NH_4OH in order to determine whether or not there are differences between these groups. No one group was used as a control for the other groups. There were no significant differences between these control/solvent groups ($0.2>P>0.1$).

Treatment group	Tumour DNA (mg/kg of tumour)			Tumour protein (mg/kg of tumour)		
	mean	sem	n	mean	sem	n
untreated	0.97	0.02	10	88.38	0.42	10
DMSO	0.95	0.02	9	86.88	1.18	9
PBS/NH_4OH	0.95	0.04	3	87.30	1.41	3
15 µg/kg sCCK-8	0.93	0.02	9	86.11	1.27	9
30 µg/kg sCCK-8	0.97	0.04	6	80.00	0.60	6
100 µg/kg sCCK-8	0.94	0.04	9	87.05	1.27	9
15 µg/kg nsG-17	0.96	0.04	9	87.05	1.27	9
30 µg/kg nsG-17	0.93	0.03	12	86.51	0.89	12
100 µg/kg nsG-17	0.96	0.02	12	86.20	1.08	12
1 mg/kg L-364,718	0.99	0.03	8	87.31	0.93	8
1 mg/kg L-365,260	0.97	0.02	7	88.20	0.34	7
1 mg/kg L-740,093	0.96	0.01	8	87.41	1.30	8
1 mg/kg RPR-X	0.96	0.02	7	86.89	1.73	7
20 mg/kg JB93182	1.01	0.09	2	88.35	1.45	2
JB93182 vehicle (saline)	1.02	0.04	6	87.61	1.29	6

Table 10.3 Human pancreatic cancer xenograft DNA and protein concentrations were unaffected by sCCK-8, nsG-17 and their antagonists.

The *in vivo* data show a block of groups involving the three variables, agonist (sCCK-8/nsG-17), dose of agonist and solvent (DMSO or NH_4OH) as shown in Table 10.4. Hence, there are 14 groups in this 3 factor experiment and another 7 groups including the antagonists which are considered separately. If there were equal numbers per group and had the data been normally distributed then a '3-way ANOVA-with-replication' would have answered whether there were any significant solvent, agonist or dose effects and whether there were any interactions. To date a non-parametric version of the 3-way ANOVA which can accommodate the unequal numbers is not available. Hence, the data have been analysed in separate blocks. Comparing the solvents with all agonists (*i.e.* 0.227 vs. 0.476) shows that the agonists give more than twice the effect of the solvents.

	Tumour weights (g)					
	DMSO		**PBS/NH_4OH**			
	0.875	0.000	0.050			
	0.059	0.000	0.000			
	0.000	0.666	0.000		**0.227**	<u>**0.227**</u>
	0.397	0.130	0.000			(n=18)
	0.493	0.328	0.070			
	0.137	0.676	0.199			
	0.313		**0.054**			
sCCK-8	0.294	0.144	0.997	0.193		
15 µg/kg	1.497	0.000	0.135	0.021	**0.389**	
	0.000	0.000	0.512	0.869		
	0.323		**0.455**			
30 µg/kg	0.044	0.576	1.186	0.000		
	1.350	0.000	0.031	0.000	**0.399**	<u>**0.446**</u>
	1.206	0.000	0.000			(n=35)
	0.529		**0.241**			
100 µg/kg	0.464	0.236	0.000	1.180		
	2.098	0.231	0.000	0.878	**0.547**	
	0.220	0.224	1.029	0.000		
	0.579		**0.515**			
	0.477	(n=18)	**0.414**	(n=17)		<u>**0.476**</u> (n=69)
nsG-17	0.000	0.018	0.961	0.000		
15 µg/kg	0.338	1.456	0.523	0.117	**0.500**	
	0.930		1.042	0.090		
	0.548		**0.456**			
30 µg/kg	0.144	0.103	0.248	0.565		
	0.293	0.506	0.163	0.203	**0.278**	<u>**0.506**</u>
	0.178	0.255	0.165	0.511		(n=34)
100 µg/kg	0.901	2.263	0.385	0.255		
	0.135	0.213	0.277	0.292	**0.764**	
	1.671		0.375	1.640		
	1.037		**0.537**			
	0.588	(n=16)	**0.434**	(n=18)		
	0.473		**0.370**			<u>**0.424**</u>
	(n=34)		(n=35)			(n=87)

Table 10.4 The weights of the xenografts from agonist treated and untreated groups in the two vehicles arranged for statistical analysis.

Analysis of the individual data shows that there are no values greater than 1.0 in the solvent groups (out of 18) but 12 out of 69 are greater than 1.0 in the agonist groups. To determine whether this is simply by chance or not, the data was analysed statistically using Chi-square tests. The Chi-square techniques involve analysis of the data in the form of frequencies and are often arranged as "2 x 2 contingency tables".

Tumour weights were classified as <1.0g or >1.0g. Hence, for solvents there were 18 and 0 cases respectively while for agonists there were 57 and 12 cases respectively. The Chi square value was 0.2<P>0.1. Re-analysing the data using those values greater or less than twice the "global average" of 0.424g (*i.e.* lower or greater than 0.848g) gave 17 and 1 cases for solvents respectively, while for agonists there were 51 and 18 cases respectively. The Chi-square value was P 0.05<P>0.02. One further analysis using results lower and greater than the "global average" gave 14 and 4 cases for solvents respectively and for agonists gave 44 and 25 cases respectively. The Chi-square value was 0.5<P>0.3. It is important to note here that data "snooping" by selection procedures can give an answer that one is looking for. One is more likely to find a difference when the dividing lines are extreme (high or low). The results here indicate that globally there may be a real difference between solvents and agonists. In order to confirm this, the experiment needs to be repeated several times to increase the numbers. In general, one rule of thumb for statistical analysis states that the greater the departure of the population distribution from normal, the larger the sample must be. Some statisticians require a sample size of 30 or more.

An inspection of the effects of the agonists (*i.e.* averages of 0.446 and 0.506 both vs. 0.227) suggests that there is no difference between sCCK-8 and nsG-17. This was confirmed by carrying out Chi square "3 x 2" contingency table" tests. The solvents were compared with gastrin and CCK. The same block groups as above were analysed. Tumour weights that were either <1.0g or >1.0g for solvents were 18 and 0 cases respectively, for sCCK-8 were 28 and 7 respectively and for nsG-17 were 29 and 5 cases respectively. The Chi square value was 0.2<P>0.1. Re-analysing the data using those values greater or less than twice the "global average" of 0.424g for solvents were 17 and 1 cases respectively, for sCCK-8 were 25 and 10 respectively and for

nsG-17 were 26 and 8 cases respectively. The Chi square value was 0.2<P>0.1. The final analysis using results lower and greater than the "global average" gave 14 and 4 cases for solvents respectively, 22 and 13 cases for sCCK-8 respectively and 22 and 12 cases for nsG-17, respectively and the Chi square value was 0.7<P>0.5. These results indicate that there was no significant differences in the tumour weights between the solvent (vehicle), sCCK-8 or nsG-17 treated groups.

A sub inspection seems to rule out the evidence of a dose-related effect of the agonists (assuming there was an effect primarily) as judged by the marginal averages for each dose level. Nonetheless, it is interesting to note that the higher doses of the agonists are associated with higher averages. Statistical analysis is not feasible on these groups consisting of n=6 or less as the distribution is not normal.

Looking at the difference for the two solvents overall shows a small ratio (i.e. 0.473 vs. 0.370, n=34 and 35 respectively). Also for the marginal averages across the two agonists (0.477 vs. 0.414 for sCCK-8 and 0.588 vs. 0.434 for nsG-17) there does not appear to be a marked influence. This is important because it is in keeping with the conclusion below that there was no statistically significant effect of PBS/NH_4OH vehicle. Chi-square analysis by "2 x 2 contingency tables" for sCCK-8 and nsG-17 dissolved in the two vehicles were carried out for tumours of weights lower and greater than the global average of 0.424g. Hence, for sCCK-8 there were 12 and 6 cases in DMSO, respectively and 10 and 7 cases in PBS/NH_4OH, respectively. The Chi square value was 0.95<P>0.9. With nsG-17 there were 9 and 6 cases in DMSO, respectively and 12 and 6 cases in PBS/NH_4OH respectively and a value of P>1.0 was obtained.

The effects of the vehicle PBS/NH_4OH were compared with the untreated group using the Mann-Whitney test. The nonparametric Mann-Whitney test based on the ranks of the numbers rather than their absolute magnitudes yielded a value of 0.1<P>0.05 between the untreated and PBS/NH_4OH groups. It is also interesting to note that 3 mice from the 6 treated with PBS/NH_4OH showed regression of the tumour at the end of the study.

The effects of the antagonists L-365,260, L-740,093, devazepide and RPR-X were compared with the DMSO solvent control utilising the Kruskal-Wallis analysis. This test yielded a P value of >1.0 indicating no significant differences between the various groups. It is interesting to note the JB93182 compound yielded 2 tumour weights of 0.117 and 0.610 and 4 mice had no tumours. Whether this response is due to the effect of the antagonist cannot be concluded since the numbers in the group are too low. However, compared to the vehicle control (saline) the data suggest that this compound may have had an inhibitory effect on the tumour growth at the high concentration of 20 mg/kg.

The DNA and protein content of the tumours were unaffected by either agonist or antagonist treatment (see Tables 3 and 4 in appendix II, p294-295). Interestingly the DNA and protein data fit a normally distributed population. Thus the data were analysed by using the unpaired Student's t test. The spleen weights from the treated and untreated mice also fitted a normal distribution, hence the Student's unpaired t test was used for spleen data analysis. The presence or absence (cases where the tumour regressed) of the tumour in the various treated and untreated groups was not significantly related to the weight of the spleens (see Table 5 in appendix II, p296).

10.5 Discussion

The xenograft of the Mia PaCa-2 cell line induced a palpable tumour at a faster rate than the other tumour cell line xenografts. This is not surprising since the Mia PaCa-2 cell line has a faster doubling rate than the other three cell lines investigated *in vitro* and *in vivo*. Published literature on the growth of these cell lines also indicate that Mia PaCa-2 (Yunis *et al.*, 1977) is the fastest growing cell line, followed by BxPc-3 (Tan *et al.*, 1986), Panc-1 (Lieber *et al.*, 1975) and the slowest growing of the four is Hs766T (Owens *et al.*, 1976) consistent with our results.

The histopathology results also confirm that the xenografts have the same phenotype as the cell lines from which they were derived as one would expect (Yunis *et al.*, 1977; Tan *et al.*, 1986; Lieber *et al.*, 1975; Owens *et al.*, 1976). Xenografts in SCID and nude mice are known to retain the morphological characteristics of the tumour/tumour cell line xenografted (Taghian & Huang, 1995).

The finding that the nude mice developed tumours before the SCID mice may reflect the greater residual immune activity in SCID mice. Nude mice have been demonstrated to have some residual T cells (Taghian *et al.*, 1993) and SCID mice have been shown to possess natural killer cells, macrophages and other haemapoietic cell lineages which are not affected by the autosomal recessive SCID mutation (Dorshkind *et al.*, 1985).

The results from our experiments indicate that sCCK-8 and nsG-17 had no effect on pancreatic tumour growth *in vivo*. The lack of effect of sCCK-8 on the human pancreatic cancer xenograft growth has been shown by a number of research groups using pancreatic cancer xenografts from various human cell lines. For example no effect on growth was shown by Hudd *et al.* (1989) with 50 µg/kg sCCK-8 on Mia PaCa-2 and Panc-1 xenograft growth, Maani *et al.* (1988), with caerulein (CCK analogue) and Nio *et al.* (1993) with 25 µg/kg of CCK-8 showed an inhibitory effect on one human pancreatic cancer xenograft and no effect on another in nude mice using cell lines PC-YY and PC-T1 respectively. Stimulatory effects have also been reported on either the same or different human cell line xenografts. For example Smith *et al.*

(1990a, 1991) showed stimulation with 15 μg/kg of CCK-9, of the SW-1990 xenograft and Upp *et al.* (1987) demonstrated the stimulation of growth of a CCK receptor positive human pancreatic cancer cell line xenograft with caerulein but no effect on the CCK receptor negative xenograft.

The literature indicates the importance of establishing the CCK receptor status of the human pancreatic cancer to be investigated. We found a low level of CCK-BR in the Mia PaCa-2 cell line *in vitro* compared to the NIH3T3CCK-BR cells. This could be one reason for the lack of effect of the agonists on tumour growth.

Smith *et al.* (1990a, 1991) demonstrated that the Mia PaCa-2 and SW-1990 cells respond to CCK-8 *in vitro* as well as *in vivo*. They showed using ligand binding that these cell lines demonstrated the presence of CCK-BR and absence of CCK-AR assays (1994).

Recently, Smith *et al*. (1995) have shown the involvement of the CCK-BR using the human Panc-1 cell line xenograft where 1 mg/kg of pentagastrin stimulated growth which was inhibited by the CCK-BR specific antagonist, L-365,260. They showed that pentagastrin increased tumour weight and DNA but not protein content of the Panc-1 xenografts. Our results show no significant effect on DNA or protein levels in the xenografts as would be expected since there was no significant increase in the tumour weights. Moreover, histopathology studies showed no increase in cell size within the tumour.

These differences in results in our laboratory to the work of other researchers can be explained by i) alteration of CCK-R in Mia PaCa-2 cells not related to growth, ii) low doses of sCCK-8 and nsG-17, iii) method and site of injection, iv) frequency of drug administration to accommodate for the variation in half lives of these agonists compared to the agonists used by other research groups, and v) duration of the study. The dose used for the various agonists varies from one research group to another. Smith *et al.* (1990a, 1991) used a final concentration of 15 μg/kg of CCK-9 dissolved in 0.15 M sodium chloride while further dilutions were carried out in 1% bovine serum albumin.

The CCK-9 was administered twice daily subcutaneously in 2% gelatin in order to prolong the absorption. The mice were treated for 20 days following the formation of a tumour from the xenografted cell line. Hudd *et al.* (1989) also used sodium chloride vehicles for the administration of 50 µg/kg of sCCK-8 and the treatment was continued for 15 days. Their drug injection site was different, being carried out intraperitoneally and twice daily, but no effect was observed in the tumour weights or DNA and protein content. The work done by Smith *et al.* (1995) on the Panc-1 xenograft with 1 mg/kg pentagastrin was carried out for a period of 24 days following the development of the tumour. The pentagastrin was dissolved in 0.05 M NH_4OH and further diluted in saline. The injections were administered twice daily in gelatin. It has been reported that a considerably higher dose of pentagastrin is required to obtain the same physiological effects as gastrin-17 or CCK (Majumdar & Goltermann, 1979). Pentagastrin is 10 times less potent than gastrin-17 in displacing radiolabelled gastrin from rat gastric mucosa gastrin receptors (Takeuchi *et al.*, 1980), explaining the high dose of pentagastrin used by Smith *et al.* (1995). The differing results for the *in vivo* studies compared with the work of Smith *et al.* (1995) can be due to the above mentioned reasons and also due to the use of a different cell line.

Effects of CCK-R antagonists on xenografts of human pancreatic cancer

Our studies also indicate that the various antagonists investigated had no effect on Mia PaCa-2 xenograft growth. This is further confirmed by the absence of any effects on the DNA and protein levels in the treated and untreated tumours. Other research workers have shown the effects of CCK-AR antagonists on xenografts of human pancreatic cancers (see next paragraph) and recently Smith *et al.* (1995) reported the inhibitory effect of a CCK-BR antagonist L-365,260, on the growth of Panc-1 xenografts.

Morimoto *et al.* (1993) showed inhibitory effects of the CCK receptor antagonist loxiglumide on the growth of a human pancreatic cancer cell line xenograft, PC-HN. This cell line was also shown to possess CCK receptors using ^{125}I-CCK-39. They suggested that loxiglumide inhibits human tumour growth by other mechanisms since

the antagonist also inhibited the growth of human pancreatic cancer cell lines that lacked CCK receptors. Later, Nio *et al.* (1993) also showed that loxiglumide had an inhibitory effect on the growth of these human pancreatic cancer xenografts and this inhibition was reversed by a trypsin inhibitor FOY-305. They suggested that the inhibitory action of loxiglumide on pancreatic tumour growth may be due to its effects on proteolytic enzymes found in the lysosomes of the pancreatic cells rather than an inhibitory effect on CCK receptors. Furthermore, the CCK-AR antagonist L-364,718 was shown to have no effect on the growth of PC-HN xenograft *in vivo* (Morimoto *et al.*, 1993).

Maani *et al.* (1988) showed that L-364,718 inhibits the the growth of a human pancreatic cancer xenograft, PGER, and that this inhibition was reversed by caerulein administration. The subcutaneously implanted tumour was shown to be CCK-R positive. They suggested this effect was due to the involvement of endogenous CCK as CCK alone had no effect on the tumour growth. Smith *et al.* (1990b) reported that L-364,718 decreased tumour weight, DNA and protein content of human pancreatic cancer cell line (SW1990) xenografts. Later the same group (Smith *et al.*, 1995) showed that L-365,260 inhibited Panc-1 xenograft growth, also resulting in a decrease in tumour weight, DNA and protein content.

The apparent lack of effect of the CCK-R specific antagonists in our laboratory may be due to several reasons i) CCK-R not related to pancreatic tumour growth, ii) the lack sufficient CCK receptors in the xenografted cell line and Singh *et al.* (1991) have shown the loss of CCK receptor binding sites with increasing passage number of the pancreatic cancers, iii) The variation in the dose regime (as mentioned previously with agonists). Smith *et al.* administered the CCK-AR antagonist (L-364,718) twice daily at 2 mg/kg and the CCK-BR antagonist (L-365,260) twice daily at a final dose of 1 mg/kg. It is also important to note that the lack of effect of L-364,718 both in our laboratory and in the work done by Morimoto *et al.* (1993) may indicate the lack of a role of CCK-AR in the growth of human pancreatic cancers. The effects of L-364,718 (devazepide) have been studied in 18 patients with advanced pancreatic carcinoma (Abbruzzese *et al.*, 1992). The subjects received 5 mg of L-364,718 orally, twice daily

for four weeks. Some of the patients received treatment for an additional 4 or 8 wecks at a dose of 10 mg twice daily. The study failed to demonstrate any impact of L-364,718 on tumour progression, the level of pain experienced or nutritional parameters. Several of the patients experienced toxic reactions even at the lower dose, although this was only about 1/25 of the dose used by Smith *et al.* (1990b) in mice. One of the patients did not complete the study because of abdominal cramps, one required dose reduction because of nausea, vomiting, abdominal cramps and two others required treatment for abdominal cramps. The dose of L-364,718 used by Smith and co-workers in mice is very high compared to the human studies, exceeding the dose used in humans by 25 fold. The inhibitory effects of L-364,718 on SW1990 xenografts (Smith *et al.*, 1990b) may be explained by toxic effects of this antagonist at such high doses. This compound has now been withdrawn from the clinic due to its toxicity even at low doses.

Since some of the CCK-BR antagonists used in these studies are novel there is a lack of information about the properties of these compounds. In these experiments the doses of the antagonists were chosen by extrapolation from published literature by Smith *et al.* (1990b), Abbruzzese *et al.* (1992) and Maani *et al.* (1993) in mice and humans. This does raise a number of questions such as the pharmacokinetics of the compound in mice. It may be that the half life of the antagonist *in vivo* is short, thus requiring either a higher dose or more frequent injections of the drug in order to maintain the concentration. Statistically the vehicle does not have any effects on the action of these antagonists as suggested by the results. Moreover, this is confirmed by the *in vitro* data obtained with these antagonists in the NIH3T3CCK-BR cells. Maani *et al.* (1988) Nio *et al.* (1993) and Smith *et al.* (1995) used the same NH_4OH vehicle (10% higher concentration) and did not report any significant differences between the control and the vehicle treated groups.

On inspection there seems to be an interesting finding in the NH_4OH and JB93182 group (Table 10.2), but with a larger sample number the data in these groups may look like the rest of the data. Scanning the other groups in Table 10.4 one notices that the mid dose of sCCK-8 with the PBS/NH_4OH solvent, that 4 out of 5 results are zero or

near to zero and there is one large figure and likewise with the lowest dose of sCCK-8 using DMSO as solvent. In such a case one is in danger of being a victim of a type II error. This occurs when we conclude that no significant differences exist when they do and the only way that this can be substantiated is by increasing the n number. However as already mentioned it is unlikely that this is the case since the pancreatic cancer cell line used in this study has a low number of CCK receptors and thus not responsive to either CCK agonists or antagonists.

Spontaneous tumour regression observed in our *in vivo* studies has not been reported by any of these workers in the control groups of nude mice bearing pancreatic carcinoma xenografts. However, tumour regression has been reported to occur in both SCID and nude mice. Several reports have demonstrated residual immune reactivity by the nude mouse against the xenografts (Rofstad, 1989; Zietman *et al.*, 1988; Taghian *et al.*, 1993). Thus the observation of spontaneous tumour regression in our laboratory can be explained by this residual immune reactivity. The *in vivo* studies carried out by Smith *et al.* (1990, 1995) were 35 days long whereas our studies were 64 days long. Recently Taghian and Huang (1995) have reported that by the time a xenograft has grown to a specific size at which it can be treated, it may well have produced a host reaction which although not strong enough to cause tumour regression will nevertheless influence regrowth after the tumour has undergone cytoreduction. Thus the endpoint of an *in vivo* study is highly critical. Future suggestions would include i) reducing the length of the study (in order to minimise the activation of the residual immune reactivity) and ii) whole body irradiation of the mice in order to depress the residual immunity.

In conclusion it is difficult to decipher a role for CCK-R in the growth of human pancreatic cancer from these experiments and the results should be repeated using larger groups.

CHAPTER II

IDENTIFICATION OF KINASES INVOLVED IN PANCREATIC CANCER DEVELOPMENT

CHAPTER 11

11.1 Background

Since the discovery of tyrosine-specific phosphorylation by pp60$^{v\text{-}src}$, members of the protein tyrosine kinase family have been increasingly identified over the last decade (Hunter, 1987; Hanks *et al.*, 1988). This large class of enzymes can be divided into two major subgroups: the receptor and the non-receptor families. Most tyrosine kinases mediate the response of eukaryotic cells to external stimuli and hence are a crucial component in the regulatory system that co-ordinates cell proliferation and differentiation (Cantley *et al*, 1991). Some terminally differentiated cells also express significant levels of tyrosine kinases, suggesting that these enzymes are important for the functioning of differentiated as well as growing cells. Receptor tyrosine kinases transmit the signal of natural ligands such as growth factors, hormones or differentiation factors. These kinases exhibit a similar molecular topology including a large glycosylated, extracellular domain, a single hydrophobic transmembrane region and a cytoplasmic catalytic domain (Ullrich & Schlessinger, 1990).

In principle every tyrosine kinase receptor bears transforming potential, since constitutive expression of its signalling function may override cellular control mechanisms. Oncogenic receptor versions are either characterised by structural deviations from normal counterparts or result from receptor overexpression through autocrine stimulation (Ullrich & Schlessinger, 1990). Qualitative alterations of receptor molecules range from gross deletions or recombinations affecting the extracellular region to single point mutations within the extracellular, transmembrane or cytoplasmic domain.

The non-receptor class of tyrosine kinases represent a collection of cellular enzymes that are grouped together because of their lack of extracellular sequences. A number of the non-receptor tyrosine kinases have been found to be associated with other cell surface proteins (which generally lack endogenous enzyme activity such as Janus kinases, JAKs) and are shown to be capable of facilitating cell surface initiated signal

transduction much like the receptor class of tyrosine protein kinases. Currently there are at least 24 individual non-receptor tyrosine protein kinases comprised of eight different groups. They range in size from 50 kDa for the C-src kinase (Csk) family to approximately 150 kDa for the Abl kinase family.

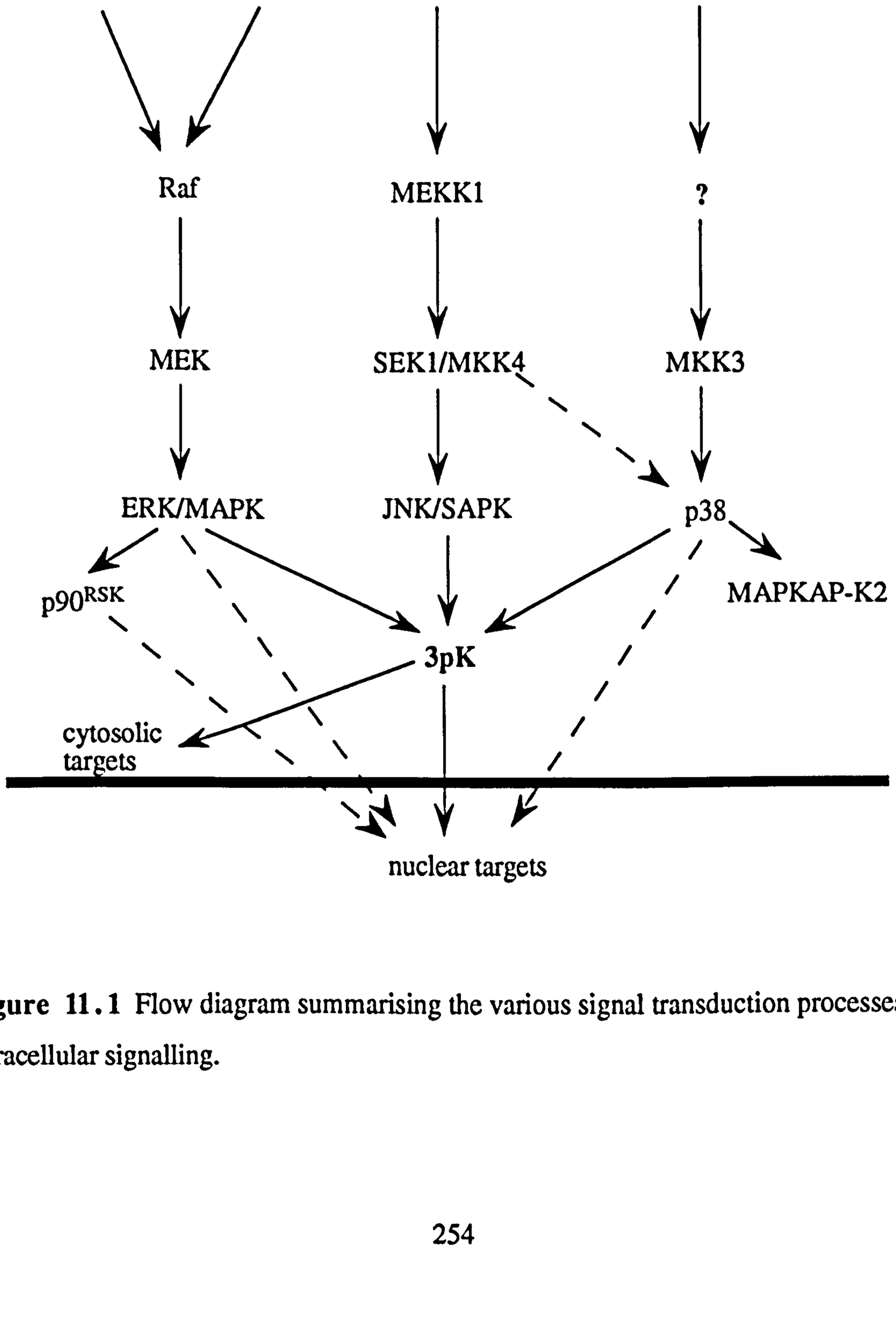


Figure 11.1 Flow diagram summarising the various signal transduction processes of intracellular signalling.

Receptor stimulation involves activation of several cytoplasmic signal transduction pathways including sequential protein kinase reactions using serine/threonine protein kinases. Mitogen activated protein kinase (MAPK, also known as ERK, extracellular regulated kinase) is a serine/threonine kinase that is stimulated by receptor tyrosine kinases and GPCR (Kahan *et al.*, 1992). Upon activation, ERK translocates into the nucleus to mediate changes in gene expression. ERK is activated by phosphorylation of both tyrosine and threonine. This reaction is catalysed by a specific threonine/tyrosine directed kinase called MAPK kinase (MEK/ERK kinase) (Crews *et al.*, 1992). In turn MEK is phosphorylated and activated by the serine/threonine protein kinase Raf (MEKK; Lange Carter *et al.*, 1993; Kyriakis *et al.*, 1992). Two additional parallel kinase cascades composed of enzymes functionally related to either Raf, MEK or ERK were discovered in mammalian cells (Cano & Mahadevan, 1995). These cascades are only poorly activated by mitogens but are strongly stimulated by cellular stress inducers. One of these recently identified cascades is preferentially trigerred by anisomycin, UV radiation and some alkylating agents and the other is activated by lipopolysaccharide, osmotic stress and heat shock leading to the activation of Jun-N-terminal kinase/stress activated protein kinase (JNK/SAPK) (Cano *et al.*, 1994; Kyriakis *et al.*, 1994) and p38RK (Rouse *et al.*, 1994) respectively (see Figure 11.1). All three kinases (ERK, SAPK and p38RK) are found in the nucleus after activation (Cavigelli *et al.*, 1995, Raingeaud *et al.*, 1995). Direct activators of JNK/SAPK and p38RK are either the dual specificity kinase SEK1/MKK4 (SAPK/ERK kinase 1) which activates both JNK/SAPK and p38RK when overexpressed or MKK3 which specifically activates p38RK.

Originally identified as MEK1/2 kinase, MEKK1 does not trigger the activation of MEK and ERK but stimulates the SAPK activator (SEK1) and thus acts parallel to raf. These activated kinases (ERK, JNK/SAPK and p38) are also known to have specific substrates in the cytoplasm. ERK is known to activate $p90^{RSK}$ MAPKAP-K1 (MAPK protein kinase-1) and p38RK activates MAPKAP-K2 (MAPK protein kinase-2; Stokoe *et al.*, 1992; Rouse *et al.*, 1994). A newly identified kinase 3pK has been demonstrated to be activated by both stress inducers and mitogens (Ludwig *et al.*,

1996). Little is known about the substrates of MAPK-K1 (ribosomal S6 kinase), MAPKAP-K2 and 3pK and the physiological effects resulting from their activation.

RSK (ribosomal S6 kinase) was originally named for its ability to phosphorylate the C-terminus of the ribosomal S6 protein. *In vivo* RSK, a serine/threonine kinase has been demonstrated to phosphorylate the serum response factor, which contributes to the regulation of serum-response factor dependent transcription (Chen *et al.*, 1993b). MAPKAP-K2 substrates include heat shock protein Hsp25 and its human counterpart Hsp 27 (Stokoe *et al.*, 1992).

The cell cycle is also controlled by serine/threonine kinases known as cyclin dependent kinases (CDKs; Hartwell & Weinert, 1989). The cell cycle is a collection of highly ordered processes that result in the duplication of a cell. As cells progress through the cell cycle, they undergo several discrete transitions. A cell cycle transition is a unidirectional change of state in which a cell that was performing one set of processes shifts its activity to perform a different set of processes. These transitions are monitored by cell cycle checkpoints. The checkpoints are a pause in the cycle that allow any damaged DNA to repair. Inhibition of CDKs is a mechanism by which some checkpoint pathways cause cell cycle arrest, *e.g.* by the tumour suppressor p21 which is a CDK inhibitor (El-Deiry *et al.*, 1993). Hence the loss of these checkpoints would result in genomic instability and has been implicated in the evolution of normal cells into cancer cells.

The diversity of the kinase signalling pathway(s) is complex and knowledge about the mechanisms is constantly expanding. Since kinases play an important role in cell growth and differentiation, they are an important and an attractive target for cancer therapy.

11.2 Aims

The aim of this study was to identify kinases potentially involved in the development of pancreatic cancer.

11.3 Methods

11.3.1 *RT-PCR*

The methods are described in Chapter 6. PCR was carried out using degenerate oligonucleotide primers targeting two invariant amino acid motifs within the catalytic domain of protein kinases in two human pancreatic cancers (PT1 and PT3). Each PCR product was cloned and several colonies (31 from PT1 and 15 from PT3) selected and sequenced.

11.4 Results

Figure 11.2 shows the amplification product of 220 bp using the degenerate primers targeting the catalytic domain of protein kinases. Table 11.1 shows the various protein kinases that were isolated from the two human pancreatic cancers investigated. Insulin like growth factor I receptor (IGF-IR) was isolated more frequently in comparison to the other kinases in both tumours.

Kinase	Pancreatic Tumour 1 (PT1)	Pancreatic Tumour 3 (PT3)
IGF-IR	16	6
PDGFβR	1	2
Eph	3	1
c- MET	1	-
UFO	1	-
JAK1	6	3
JAK2	1	1
c-Abl	1	1
Novel	1	1

Table 11.1 Kinases isolated from two human pancreatic cancers, PT1 and PT3.

The predicted amino acid sequence obtained from the 220 bp DNA sequence for the various protein kinases are shown in Table 11.2.

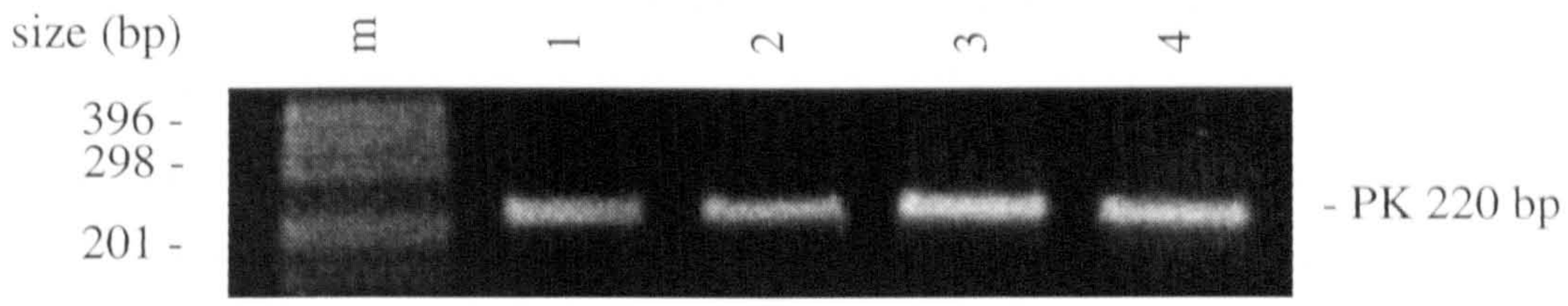


Figure 11.2 Agarose gel electrophoresis of protein kinases (PK) RT-PCR products from two human pancreatic cancers (PT1 and PT3). The size of the amplified product was 220 bp.

Lane 1: human pancreatic cancer 1 (PT1); 2: human pancreatic cancer 3 (PT3); 3,4: controls utilising cDNA from NIH3T3 and Mia PaCa-2 cells respectively; m: molecular markers.

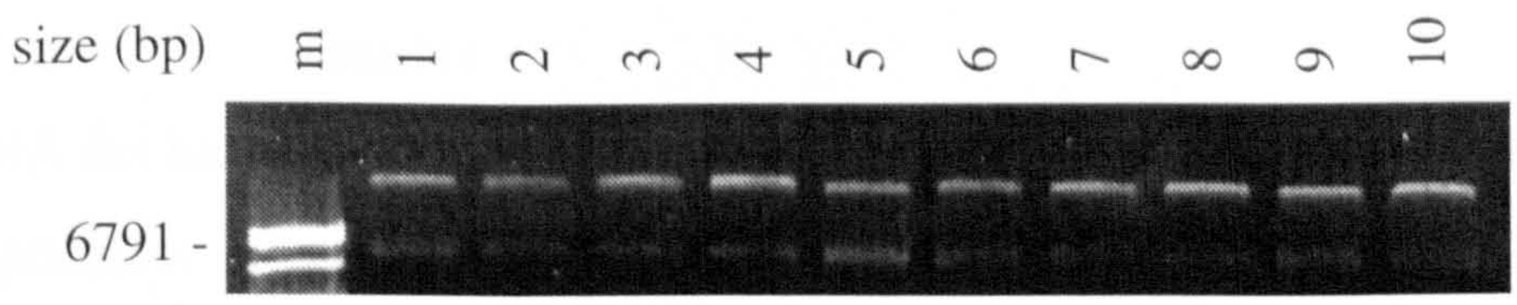


Figure 11.3 Agarose gel electrophoresis showing equal loading of protein kinase DNA used in dot blots.

Protein kinases were loaded as follows: Lane 1: Eph; 2: PDGFβR; 3: UFO; 4: c-abl; 5: c-met; 6: JAK1; 7: JAK2; 8: IGFR-1; 9,10: novel kinase; m: molecular markers.

A novel kinase was isolated from both PT1 and PT3. This novel kinase is a serine threonine kinase as demonstrated by the conserved amino acid sequences in the catalytic domain (Table 11.2). The alignment was made by eye and the gapping introduced into the sequences in order to optimise the position of the similarities between the kinases isolated. This alignment clearly demonstrates the overall similarity among the catalytic domains. The catalytic domains are not conserved uniformly but rather consist of alternating regions of high and low conservation.

11.5 Methods

11.5.1 *DNA dot-blots*

The methods are described in 6.11. PCR was carried out using the degenerate oligonucleotide primers from cDNA synthesised from 4 human pancreatic cancers (PT1-PT4), 3 normal human pancreatic specimens (histologically diagnosed as normal, PN1-PN3) and four human pancreatic cancer cell lines (Mia PaCa-2, BxPc-3, Capan-1 and Hs766T. The 220 bp amplified product was cleaned and used as a template for the synthesis of a radioactively labelled probe. The labelled probe was hybridised overnight with the kinase dot blots prepared using the DNA obtained from each isolated kinase from PT1 and PT3. The dot blots were washed the following day and Kodak film was exposed to the blots overnight before development.

11.6 Results

DNA dot blots of the kinases isolated were used to assess the levels of specific kinase expression. All normal and malignant pancreas and human pancreatic cancer cell lines showed the novel kinase and IGF-IR to be highly expressed relative to the other 7 kinases in each specimen. Equal loading of the protein kinase DNA blotted was confirmed by agarose gel electrophoresis as shown in Figure 11.3. The β-actin negative control did not hybridise to the probes synthesised, showing that the hybridisation was kinase specific. Furthermore when comparing the 220 bp kinase sequence from each kinase used, there was only 56% similarity at the nucleic acid sequence level when the primer sequence was removed, with no long range sequence similarity. Thus under the hybridisation conditions used one would not expect any cross-hybridisation between the different kinases.

SUBDOMAIN	VI	VII	VIII	IX
	PTKI [V/I]HRDL N	KI DFG	P W APE	PTKII DVWSFG
IGFR-1	VHRDLAARNCMVAEDF	TVKIGDFGMTRDIYETDYYRKG	GKGLLPV RWMSPESLKDGVFFYSSDVWSFG	
PDGFβR	VHRDLAARNVLICEGK	LVKICDFGLARDIMRDSNYISK	GSTFLPLKWMAPESI FNSLYTTLSDVWSFG	
EPH	IHRDLLAARNILVNQNL	CCKVSDDFGLTRLLDDFDGTYETQ	GGKIPI RWTAPEAI AHRIFTTASDVWSFG	
c-MET	VHRDLAARNCMLDEKF	TVKVADFGLARDMYDKEYYSVHNK	TGAKLPVKWMALESL QTQKFTTKSDVWSFG	
UFO	IHRDLAARNCMLNENM	SVCVADFGLSKKIYNGDYYRG	QIAKMPVKWIAIESLA DRVYTSKSDVWSFG	
JAK1	IHRDLAARNVLVESEH	QVKI GDFGLTKAIETDKEYYTVK	DDRDSPVFWYAPECLMQSKFYIAYDVWSFG	
JAK 2	IHRDLATRNILVENEN	RVKI GDFGLTKVLPQDKEYYKVK	EPGERPI FWYAPESL TESKFSVASDVWSFG	
c-ABL	IHRDLAARNCLVGENH	LVKVADFGLSRLMTGDTYTAH	AGAKFPIKWTAPESL AYNKFSIKSDVWAFG	
NOVEL	IHRDLKPTNILLGDEG	QPVLMDLGSMNQACIHVEGSRQALTLQDWAAQPCTISYRAPELFSVQSHCVIDKRTDVWSLG		

Table 11.2 The predicted amino acid alignment of the catalytic domain of all the protein kinases found in two human pancreatic cancers. Conserved amino acids are shown in bold and have been used to align the protein kinases on the predicted amino acid sequences. In subdomain VI (underlined), AAR or RAA is characteristic of a tyrosine kinase where KXX indicates a serine/threonine kinase. In subdomain VIII (underlined), W-T/M-A-P-E is highly conserved in tyrosine kinases whereas serine/threonine kinases show low conservation F-X-A-P-E. The novel kinase has 28% homology to the ribosomal protein S6 kinase II beta.

PT1 expresses all the 9 kinases but to varying levels as shown in Figure 11.4. 7 different kinases were isolated from PT3 and the dot blots indicate the detectable expression of 5 different kinases indicating that the kinases are expressed at different levels. The kinases PDGFβR, c-MET and JAK2 showed a low level or no expression in the 4 cancers investigated as shown in Figure 11.4 (see Table 9.1 for details of the pancreatic tumours investigated).

The pancreatic cancer cell lines show a differential pattern of expression shown in Figure 11.5. The Mia PaCa-2 cell line expressed similar levels of PDGFβR, UFO, IGF-IR and the novel kinase. The pancreatic cancer cell lines Capan-1 and Hs766T show a similar pattern of expression of the kinases with PDGFβR, IGF-IR and the novel kinase being overexpressed in comparison to the other kinases. The BxPc-3 cells show an overexpression of IGF-IR and the novel kinase with PDGFβR and c-Abl, expressed more than the other kinases in this cell line. In summary all the pancreatic cancer cell lines investigated showed an overexpression of the novel and IGF-IR kinases compared to the other 7 kinases in these cell lines.

The normal pancreatic specimens also show expression of IGF-IR as well as the novel kinase with 2 of the normals (PN2 and PN3, see Table 11.3 for details of tissues used) also showing a higher expression of PDGFβR in comparison to the other kinases within each tissue shown in Figure 11.6.

Pancreatic tissue	Sex	Age	Diagnosis
PN1	male	64	Cholangiocarcinoma
PN2	male	43	Cholangiocarcinoma
PN3	female	36	Cholangiocarcinoma

Table 11.3 Fresh pancreatic specimens used for kinase expression studies.

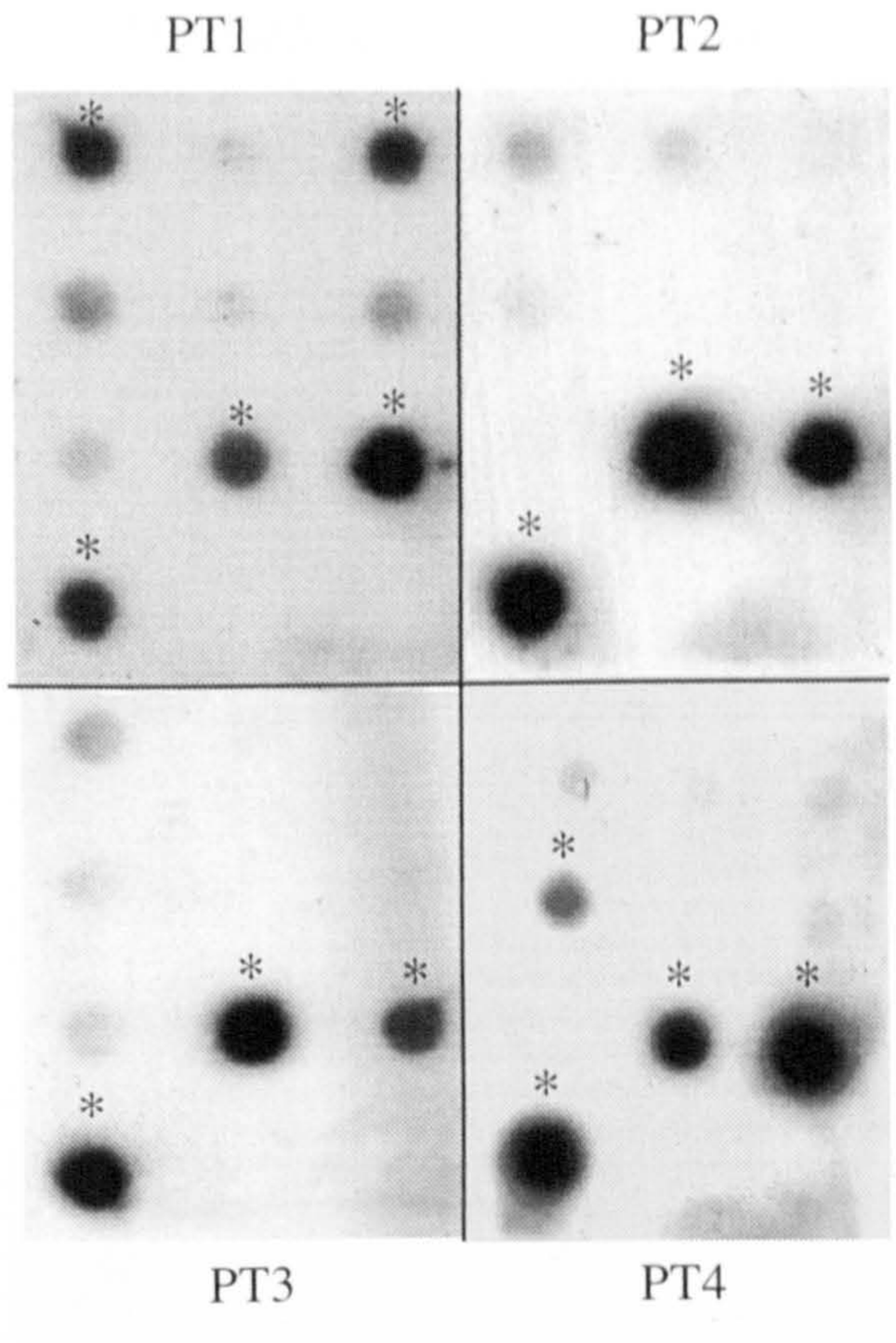


Figure 11.4 Dot blots showing the relative abundance of each protein kinase in four human pancreatic cancers.

Tumour 1: PT1, Tumour 2: PT2; Tumour 3: PT3 and Tumour 4: PT4.

Key is shown below.

Eph	PDGFβR	UFO
c-Abl	c-MET	JAK1
JAK2	IGFR-1	Novel
Novel	β-actin	

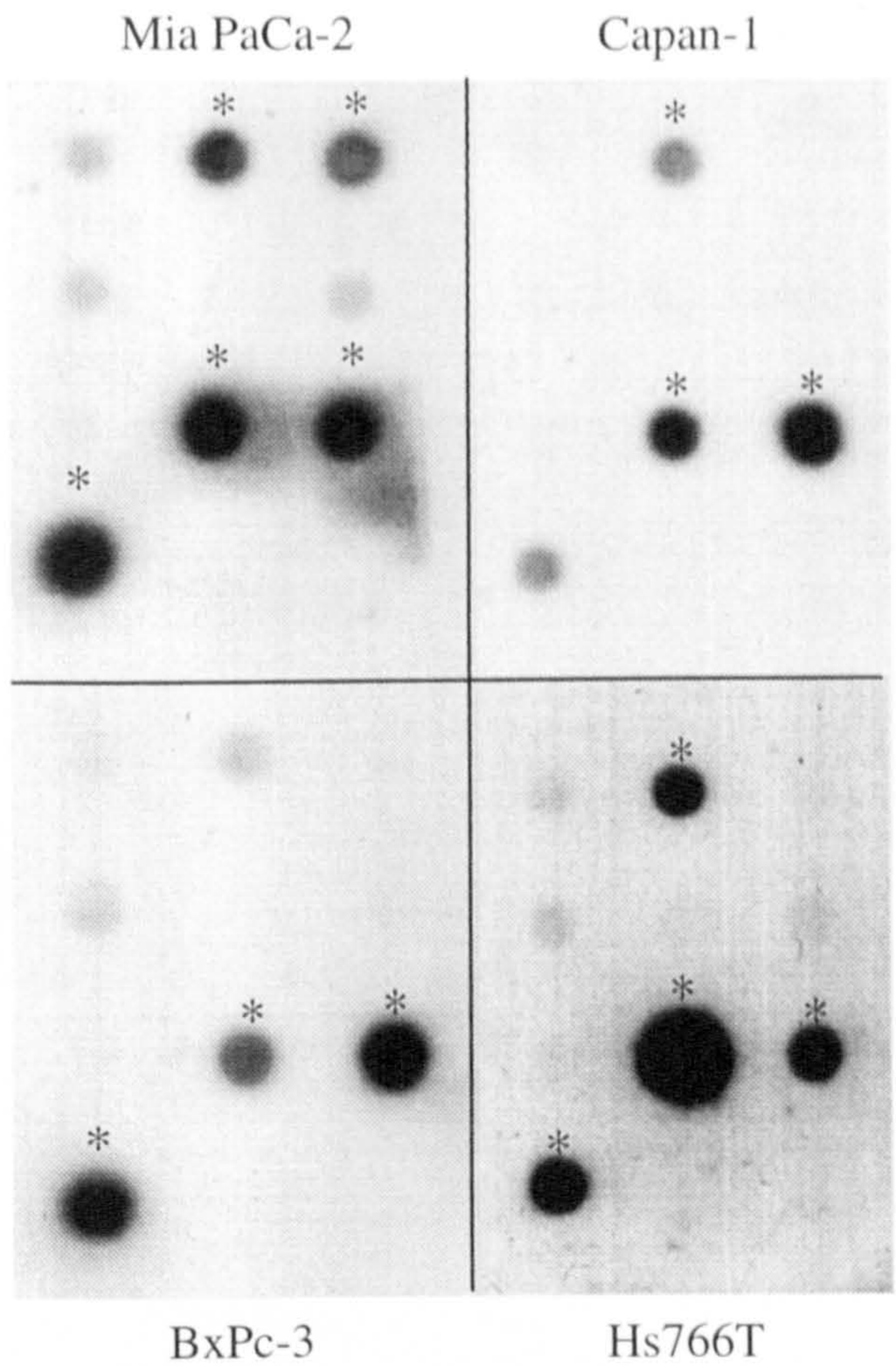


Figure 11.5 Dot blots showing the relative abundance of each protein kinase in four human pancreatic cancer cell lines: Mia PaCa-2, Capan-1, BxPc-3 and Hs766T. Key is shown below.

Eph	PDGFβR	UFO
c-Abl	c-MET	JAK1
JAK2	IGFR-1	Novel
Novel	β-actin	

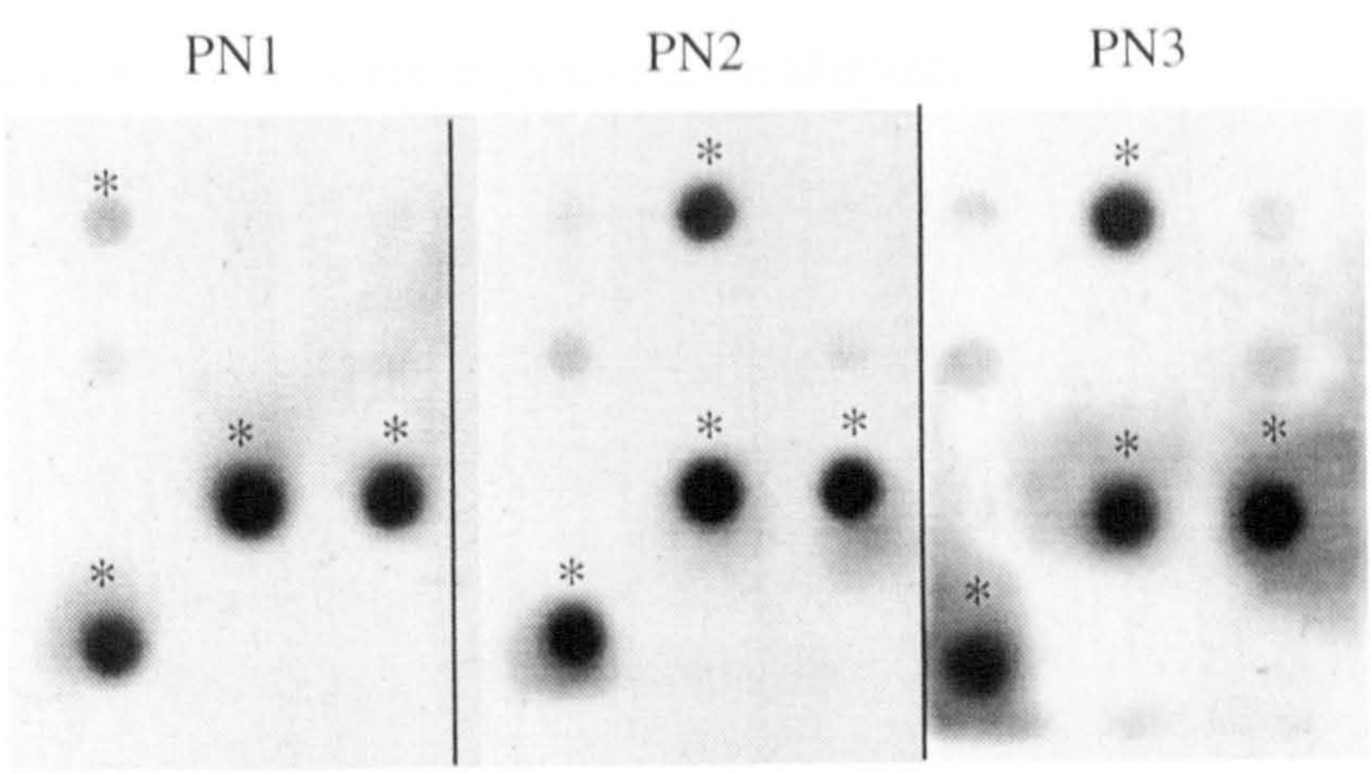


Figure 11.6 Dot blots showing the relative abundance of each protein kinase in three normal human pancreases.

'Normal' 1: PN1; 'Normal' 2: PN2; 'Normal' 3: PN3.

Key is shown below.

Eph	PDGFβR	UFO
c-Abl	c-MET	JAK1
JAK2	IGFR-1	Novel
Novel	β-actin	

Low levels of other kinases such as Eph, UFO, c-Abl and JAK1 were also detected in 2 of the normal specimens (PN1 and PN2). However, the data cannot be compared between the normal and malignant specimens as parameters such as exposure time and specific activity of the probe have to be taken into consideration. I did not have enough RNA for this comparison which would require Northern blotting.

It is important to note that this method is a semi-quantitative method and is a good indicator of expression levels but is by no means absolute.

11.7 Discussion

Table 11.2 shows highly conserved individual amino acids within the catalytic domains of each kinase which play an important role in catalysis. Subdomain VIII contains the consensus sequence APE, a conserved feature often mentioned as a key protein kinase catalytic domain indicator (Hunter & Cooper, 1986). Mutagenesis studies have shown that each residue in the APE consensus is required for activity of v-Src. The novel kinase has been demonstrated to possess the APE sequence. Sites of autophosphorylation have also been found to lie near to this site (Shoji, 1981) but their role is not entirely clear. Subdomains VI and VIII are conserved in either protein tyrosine kinases or serine/threonine kinases. It has been suggested that these domains may play an important role in recognition of the correct amino acid either serine, threonine or tyrosine (Hanks *et al.*, 1988). The sequence DLKPEN in subdomain VI is known to be a strong indicator of a serine/threonine kinase. In the novel kinase the sequence is DLKPTN while the sequence DLAARN/DLRAAN is indicative of a protein tyrosine kinase. Another highly conserved sequence among the protein tyrosine kinases with a more limited conservation among the protein serine/threonine kinases is PI/VK/RWT/MAPE in subdomain VIII. In the serine/threonine protein kinases the consensus is GT/SXXY/FXAPE. The amino acid sequence of the novel kinase also possesses this consensus site.

Insulin like growth factor-I (IGF-I) is a polypeptide that exhibits structural homology to proinsulin and exerts growth-promoting and metabolic effects (Froesch *et al.*, 1985). Its actions are dependent on the presence of specific cell surface receptors, the IGF-I receptor (IGF-IR). IGF-IR is a tyrosine kinase receptor with 70% structural homology to the insulin receptor (Ullrich *et al.*, 1986). IGF-IR has a high affinity for IGF-I and a low affinity for insulin and IGF-II (Werner *et al.*, 1991). The IGF-IR is composed of of a heterotetrameric $\alpha_2\beta_2$ subunit structure and has a ligand-stimulated tyrosine kinase activity. Unlike other tyrosine kinase receptors such as platelet derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR), the insulin receptor does not have direct associations with SH2 proteins. In contrast the activated insulin receptor phosphorylates insulin receptor substrate-1 (IRS-1) on multiple tyrosine

residues and Shc, which in turn recognise and bind to various signal transduction proteins such as PI-3- kinase, ras, and MAPK. Cells treated with insulin rapidly increase glucose uptake and lipid and glycogen synthesis but only increase DNA synthesis after a prolonged stimulation. IGF-I however, appears to be a more potent stimulator of DNA synthesis and cell growth (Randszzo *et al.*, 1990). IGF-I functions as an autocrine and a paracrine growth factor in a variety of mesenchymal and epithelial tumours (Macaulay, 1992; Daughaday, 1990) and is essential for entry into the S phase of the cell cycle (DNA synthesis, Baserga, 1995).

The dot-blots indicated that IGFR-I was highly expressed compared to the other kinases isolated in all the pancreatic specimens investigated. Pancreatic tumours have been shown to overexpress mRNA for both IGF-I and IGF-IR by Northern blot analysis confirming our findings. It has been suggested that IGF-I may play a role in the aberrant autocrine and paracrine activation of IGF-IR in pancreatic cancer as the IGF-IR was found to be overexpressed in those pancreatic cancers that had a high expression of IGF-I mRNA (Bergmann *et al*., 1995).

The c-*MET* proto-oncogene encodes a transmembrane tyrosine kinase receptor (MET) that has the capacity to modulate cell proliferation and differentiation (Cooper *et al.*, 1984). It is activated by the hepatocyte growth factor (HGF). HGF is a 105 kDa protein that is mitogenic to hepatocytes, endothelial and epithelial cells (Montesano *et al.*, 1991; Zarnegar *et al.*, 1990). The HGF action is dependent on its binding to MET, the HGF receptor. As with most receptor tyrosine kinases, MET is triggered by HGF dependent homodimerisation and cross phosphorylation on specific tyrosine residues in the intracellular carboxy terminus of the receptor. Activation of MET leads to stimulation of various intracellular signalling cascades, including PLCγ, ras, phosphotyrosine phosphatase, $pp60^{src}$, MAPK and PI-3-kinase.

Our results indicate a low level of MET expression compared to the other kinases such as IGF-IR and PDGFβR. However, c-MET has been shown to be overexpressed in pancreatic cancer (Di Renzo *et al.*, 1995) and the overexpression is associated with

increased levels of HGF mRNA (Ebert *et al.*, 1994). The importance of this upregulation is not yet determined but it may be involved in motility or invasiveness of pancreatic tumour cells (Oikawa *et al.*, 1995).

PDGF was identified over 20 years ago as a growth promoting activity in human platelets for fibroblasts, smooth muscle cells and glial cells. There are three PDGF isoforms, PDGF-AA, -AB and -BB. Two receptors for PDGF, denoted the PDGFα and PDGFβ receptor binds the PDGF isoforms with differing affinities. The PDGFβR only binds the B-chain containing PDGF isoforms. Binding of PDGF activates the receptor by dimerisation, accompanied by tyrosine phosphorylation of the receptor. The phosphorylated tyrosines bind various intracellular messengers such as, cytoplasmic kinases, Src, Fyn and Yes, Shc, PLCγ, PI-3 kinase, ras and MAPK leading to cell proliferation, cell migration, angiogenesis or membrane ruffling/cytoskeletal rearrangement depending upon cell type.

PDGF and PDGFβR have been shown to be involved in an autocrine manner in tumour growth (Nister *et al.*, 1991). Glioblastoma cell lines express both PDGFαR and PDGFβR types as well as PDGF in different ways: PDGFαR is mainly expressed in the tumour cells whereas the PDGFβR is expressed in the stroma (Hermanson *et al.*, 1988, 1992). The PDGFβR has been shown to be important in tumour development by providing the tumour with nourishment (Forsberg *et al.*, 1993). To date there has been no work carried out on PDGF receptors in pancreatic cancer.

The JAK-STAT (Janus kinase-signal transducers and activators of transcription) pathway is a newly discovered intracellular signal transduction pathway that is used by a growing number of extracellular signalling proteins resulting in either cell growth, or differentiation (Darnell *et al.*, 1994). It is now recognised that JAK-STAT pathways are activated by most cytokines (Sadowski *et al.*, 1993), growth factors that bind to receptor tyrosine kinases (Shuai *et al.*, 1993) and by the ligand induced activation of certain GPCR (*eg.* angiotensin II, Marrero *et al.*, 1995).

Ligand binding results in the dimerisation (or multimerisation) of the receptor subunits and consequent activation of one or more JAKs. JAKs are cytoplasmic non-transmembrane tyrosine kinases with a molecular weight between 125 kDa and 135 kDa. Reports vary as to whether the JAKs are constitutively associated with the receptor or associate with the receptor following ligand binding. Moreover, it is not known whether the association of JAKs with the receptor is direct. Nevertheless, JAK activation requires the integrity of a proline-rich sequence within a membrane-proximal region of the receptor subunits cytoplasmic domains.

Activated JAKs generally phosphorylate tyrosine residues on one of the receptor subunits, thereby providing sites for binding by the SH2 domains of specific STATs. The bound STATs are then phosphorylated on a single tyrosine, presumably by the receptor associated JAKs, dimerise by a high affinity reciprocal SH2-phosphotyrosine interactions and are translocated to the nucleus. Stat proteins translocate to the nucleus, bind specific DNA sequences and promote transcription. The JAK family is presently comprised of four mammalian enzymes, JAK1, 2, 3 and Tyk2 and six mammalian STAT genes have been isolated (Ihle, 1996). Different ligands specifically activate different members of the JAK and STAT families.

JAK1 was detected in both 'normal' and cancer pancreatic tissues and pancreatic cancer cell lines. However, JAK2 was only found to be expressed in 2 human pancreatic tumours and not in the 'normal' pancreatic specimens. Whether this is indicative of overexpression of JAK2 in human pancreatic cancer remains to be elucidated. With the exception of JAK3 all JAKs are expressed in most tissues. The expression of JAK3 seems to be restricted to cells of the immune system (Kawamura *et al.*, 1994) but as yet no work has been done in the expression and role of JAKs in cancer.

Eph belongs to the largest family of receptor tyrosine kinases. Eph was the first receptor to be discovered, isolated from human hepatocellular carcinoma cell line cDNA library (Hirai *et al.*, 1987). This receptor has been found to be overexpressed in lung, liver, breast and colon carcinoma (Maru *et al.*, 1990). However, no ligand for eph has yet been reported, thus restricting the studies on eph function.

The c-Abl tyrosine protein kinase appears to be a multifunctional tyrosine kinase localised to the nucleus and cytoplasm (Van Etten *et al.*, 1989). Biochemical studies suggest a role in cell cycle progression or transcriptional regulation (Kipreos & Wang, 1990). Recently c-Abl has been reported to act as a negative regulator of cell growth (Sawyers *et al.*, 1994) and cells deficient in this kinase fail to activate stress activated protein kinase (SAPK) after treatment with DNA damaging agents (Kharbanda *et al.*, 1995). An additional role of c-Abl in signal transduction is provided by the finding that the c-Abl SH3 domain associates with a protein (3BP1) through a proline-rich motif and that the 3BP1 protein possesses additional sequences that are related to other proteins with GTPase activating activity toward members of the Rho/Rac family of small G proteins (Cicchetti *et al.*, 1992, Ren *et al.*, 1993). Rho and Rac proteins have a role in membrane ruffling and in the formation of actin stress fibers as well as focal contacts suggesting that c-Abl may have an important role in regulation of the cell cytoskeleton.

c-Abl was detected in all specimens examined except in the cell line, Capan-1. This probably reflects the multifunctional role of the kinase in this tissue. However, to-date no expression studies or functional studies of c-Abl have been carried out in cancer.

UFO (unidentified function of protein)/Axl (from Greek word anexelekto meaning uncontrolled) was originally isolated from three patients with chronic myeloid leukaemia (Janssen *et al.*, 1991; O'Bryan *et al.*, 1991). This kinase has transforming characteristics which results from overexpression of Axl mRNA rather than from structural mutation (O'Bryan *et al.*, 1991). Northern-blot studies show the kinase to be expressed in human bladder and cervical cell lines and in a number of organs in the mouse such as bone marrow, ovary and spleen. Two distinct variants of the UFO/Axl receptor have been identified (Janssen *et al.*, 1991; O'Bryan *et al.*, 1991) but the significance of this is not yet known. The functional role of this kinase has not been elucidated and literature on this kinase is sparse. To date UFO/Axl expression studies in cancer have not been carried out.

The novel 220 bp DNA sequence depicts a serine/threonine kinase. The overexpression of the novel serine/threonine kinase compared to its expression in the normal human pancreatic samples makes this kinase a potential target for pancreatic cancer therapy. This finding is very interesting and needs further investigation (see future studies section 12.3 p275). This kinase may be involved in the control of cell cycle regulation as are other serine/threonine kinases and furthermore it may be specific to the pancreas.

Very little work has been reported on the regulation/disregulation of kinases in pancreatic cancer. This would be a very important field to pursue, since kinases are known to lead to cell proliferation and/or differentiation. Findings such as overexpression/novel expression or even variation in activity levels of these kinases may provide new diagnostic markers for pancreatic cancer. Moreover, these kinases may prove to be important in the development of this disease and a potential target for the treatment of pancreatic cancer.

CHAPTER 12

DISCUSSION AND CONCLUSION

CHAPTER 12

12.1 Discussion

The aim of this project was to determine the role of CCK-A and CCK-B receptors in the growth of human pancreatic cancer cells using *in vitro*, *in vivo* and molecular studies.

The *in vitro* and *in vivo* studies suggest that both the CCK-A and CCK-B receptor types do not have an important role in the growth of human pancreatic cancer. Moreover, molecular expression studies indicate a low level of expression of CCK receptors in human pancreatic cancer and their established cell lines.

The *in vitro* cell culture studies show both sCCK-8 and nsG-17 to be ineffective in the growth of human pancreatic tumour cell lines and cancers used. Similarly, the preliminary *in vivo* data does not show a role for either sCCK-8 and nsG-17 in the growth of human pancreatic cancer.

One of the many findings in the recent explosion in CCK-R research is the involvement of ras in CCK-R signalling. This questions the role of CCK-R antagonists in the treatment of human pancreatic cancer. The antagonists were shown to have an inhibitory effect on sCCK-8 stimulated growth in NIH3T3CCK-BR cells which possess a wild type ras protein, but the mutated ras protein (found in 90% of human pancreatic cancers) may by-pass the requirement for CCK-R activation in human pancreatic tumour growth. Whether the CCK-R feed through ras-independent pathways leading to cell proliferation/differentiation remain to be determined.

The kinase study showed the expression of a novel serine/threonine kinase which is overexpressed in all human normal and malignant pancreas as well as pancreatic cancer cell lines investigated. The over/novel expression or even variation in activity levels of kinases may provide new diagnostic markers/therapeutic targets for pancreatic cancer.

This thesis covers a wide area examining the role of CCK-R in pancreatic cancer ranging from molecular to *in vivo* studies. A lack of sufficient human pancreatic cancer specimens with controls was a problem for all aspects of the project. During the 3 years only 5 human pancreatic cancers with matched normal specimens and a further 4 pancreatic cancers without normal specimens were obtained from the hospitals in the South-East. More importantly as mentioned in the discussion (section 9.6), those tumours with matched normal controls were from patients with cancer at a less advanced stage and the disease may display different growth characteristics at a later stage. To overcome these problems, it would be better to work with fresh tumour tissue from theatre, having established a library of pancreatic tumours, and to focus on receptor-expression studies before pursuing growth studies *in vitro* or *in vivo*.

In order to overcome the problem of tissue shortage, cell lines were initially used as they are readily available. This thesis has highlighted the flaw in using established cell lines for research purposes mainly in growth dependent studies. The constant adaptation to the environment by the cells results in cells that are not representative of the original tumour.

12.2 Conclusion

We have not identified a role for cholecystokinin receptors in pancreatic cancer growth *in vitro*. However, their role *in vivo*, if any, remains to be elucidated but seems unlikely. The identification of a novel serine/threonine kinase which is over-expressed in human pancreatic cancer may provide a new clue to the development of this disease.

12.3 Future studies

A pancreatic cancer tissue library needs to be built up at the outset of any project using human pancreatic tissue, as one does not exist.

In order to determine the role of CCK-R in human pancreatic cancer growth transfection studies using the mouse fibroblast NIH3T3 cell line should be carried out. Transfection of NIH3T3 cells with mutant ras with either the CCK-A or CCK-B receptor would prove to be a valuable study saving time and pancreatic tissue. These experiments would involve the elucidation of ras dependent/independent CCK-R stimulated pathways leading to cell proliferation and/or differentiation. If ras-independent CCK-R stimulated growth pathways are involved then molecular, *in vitro* and *in vivo* studies can be pursued using primary human pancreatic cancers only. Initially the receptor status of the tumour should be determined using both molecular (RT-PCR and RNP assay) and radioligand binding studies. Once the receptors are detected using the binding studies then *in vitro* and *in vivo* experiments can be set up with constant monitoring of the CCK-R receptor status during the studies.

The finding of the novel serine/threonine kinase provides a very interesting area as a potential target for pancreatic cancer therapy. This kinase may prove to be overactive in cancers compared with normally associated tissues even though it was shown to be over-expressed in the 'normal' pancreatic tissues. Thus, future work would include sequencing of the the full length kinase, then transfection of the kinase into a fibroblast cell line such as NIH3T3 cells in order to elucidate the function and properties of this enzyme particularly in respect of its transforming properties and carcinogenic potential.

APPENDIX I

MATERIALS

Solutions that were autoclaved were sterilised at 121°C for 20 min.

CELL CULTURE

DMEM (low sodium bicarbonate)

13.5 g DMEM powder with 0.6 g of glutamine and 2.0 g sodium bicarbonate dissolved in 1 L of ddH_2O, medium was filter sterilised with a Millipore sterilising filter unit.

Foetal calf serum, horse serum and bovine calf serum

All serum was thawed overnight at 4°C from frozen then heat-inactivated at 56°C for 30 min before use. Foetal calf serum was purchased from JRH /biosciences and bovine calf and horse serum were purchased from Sigma.

Phosphate buffered saline

48 g of phosphate powder (Gibco BRL) was dissolved in 5 L of ddH_2O and autoclaved to give a phosphate buffered saline solution of 0.2 g/l potassium chloride, 0.2 g/l KH_2PO_4, 0.05 g/l $MgCl_2$, 8.0 g/l NaCl and 1.15 g/l Na_2HPO_4.

Trypsin

10 ml of sterile phosphate buffered saline was added to each vial of bacto trypsin powder (Difco) and filter sterilised using 0.45 μM filters. The sterilised solution was diluted 1:20 in PBS prior to use.

Cell lines

7 human pancreatic cancer cell lines, Mia PaCa-2, Panc-1, Capan-2, BxPc-3, Capan-1, Capan-2, AsPc-1 and Hs766T were purchased from the American Type Tissue Culture (ATCC,USA). One human pancreatic cancer cell line, KPan and a T cell lymphoma cell line, Jurkatts J6 were a kind gift from Dr. Darling (Department of Molecular Medicine, King's College School of Medicine and Dentistry, UK). The mouse fibroblast NIH3T3 cell lines, wild type NIH3T3 and NIH3T3CCK-BR were a

generous gift from Dr. Matsui (Kobe University, Japan).

Cell culture media

The cell culture media, DMEM and RPMI 1640 were purchased from Sigma and stored in a refrigerator.

Antibiotics

The antibiotics penicillin and streptomycin were purchased from Sigma at 1000 U/ml and stored at -20°C.

Epidermal growth factor

0.25 mg of epidermal growth factor (Sigma) was dissolved in 10 ml of sterile phosphate buffered saline and filter sterilised with a 0.45 μM filter (ICN). The solution was stored at -20°C.

Insulin

100 mg of insulin (Sigma) was dissolved in 20 ml of 0.1% acetic acid (BDH) and filter sterilised with a 0.45 μM filter (ICN). The solution was stored at -20°C.

1.4 mM Hydrocortisone

86 mg of hydrocortisone (Sigma) was dissolved in 19.15 ml of sterile ddH_2O and filter sterilised with a 0.45 μM filter (ICN). The solution was stored at -20°C. .

5 mM Isobutylmethylxanthine (IBMX)

21 mg of IBMX (Sigma) was dissolved in sterile 0.5 mM sodium hydroxide and filter sterilised with a 0.45 μM filter (ICN). The solution was stored at -20°C.

250 mM Glutamine

3.65 g of glutamine (Sigma) was dissolved in sterile ddH_2O and filter sterilised with a 0.45 μM filter (ICN). The solution was stored at -20°C.

Peptide agonists

1 mg of the octapeptide sulphated cholecystokinin (sCCK-8) and non-sulphated gastrin I (nsG-17) were dissolved in sterile 0.01M ammonium hydroxide solution. Further dilutions were made in sterile phosphate buffered saline. The agonists were stored at -20°C. These peptide agonists were purchased from Cambridge Research Biochemicals.

Antagonists

1 mg of all the antagonists except L-740,093 were dissolved in 100% DMSO (Sigma). 1 mg of L-740,093 was dissolved in sterile phosphate buffered saline. The antagonists were stored at -20°C. Further dilutions of the antagonists (except L-740,093) were carried out in a solution consisting of 40% DMSO and 60% phosphate buffered saline. The antagonists L-364,718, L-365,260 and L-740,093 were a generous gift from Merck Sharpe and Dohme. RPR-X, was a gift from Rhone Poulenc Rorer and the antagonists CI988 and CR 1409 were a gift from Parke-Davis and Rotta laboratories respectively.

Plastic ware

All the disposable plastic materials used such as 24 well plates, falcon tubes, cryovials were purchased from Greiner (UK) unless stated otherwise.

FAK PHOSPHORYLATION STUDIES

Bradford solution

100 mg of coomassie blue (Sigma) was dissolved in 50 ml of ethanol (BDH) followed by the addition of 100 ml orthophosphoric acid. The volume was made upto 1 litre by adding 850 ml of distilled water. The solution was stored away from light at 4°C.

Coomassie blue stain

50% methanol (v/v) (BDH), 0.05% (v/v) coomassie brilliant blue R-250 (Sigma), 10% (v/v) acetic acid (BDH), 40% distilled water.

Destaining solution

5% methanol, 7% acetic acid (methanol), 88% distilled water .

DMEM (low sodium bicarbonate)

13.5 g DMEM powder with 0.6 g of glutamine and 2.0 g sodium bicarbonate dissolved in 1 L of ddH_2O, medium was filter sterilised with a Millipore sterilising filter unit.

Enhanced Chemiluminescence Kit

The ECL kit was purchased from Amersham and stored at 4°C in darkness.

Immunoprecipitation buffer (2x)

2% Triton -X-100 (BDH), 300 mM sodium chloride (BDH), 20 mM Tris pH 7.4, 2 mM EDTA (BDH), 2 mM EGTA (BDH), 0.4 mM sodium vanadate (Sigma), 0.4 mM PMSF (Sigma, dissolved in 100% ethanol), 1.0% NP-40 (BDH), 20 mM sodium fluoride (Sigma).

Phosphate buffered saline

48 g of phosphate powder (Gibco-BRL) was dissolved in 5 L of ddH_2O and autoclaved.

Running Buffer (10x)

15.5 g Tris (BDH), 72 g glycine (BDH), 5 g SDS (BDH) dissolved in 1 L of distilled water.

Sample buffer for SDS protein gel electrophoresis

1.1 ml of sample buffer was prepared by adding 200 µl 10% SDS, 240 µl 40% glycerol (Sigma), 80 µl 1 M Tris-Cl (pH 6.8), 10 µl 0.2% bromophenol blue (Sigma) 460 µl of sterile distilled water. This was stored at -20°C. 100 µl of 1M DTT (BDH) was added fresh on day of use to 1.1 ml of sample buffer.

Separating gel (6.5%)

6.5 ml 40% acrylamide (BDH), 3.3 ml 2% bis-acrylamide (BDH), 15 ml 1.5 M Tris

pH 8.8, 200 µl 20% SDS, 11.6 ml distilled water , 200 µl 20% ammonium persulphate (BDH), 40 µl TEMED (BDH).

Stacking gel (3.5%)

1.5 ml 40% acrylamide, 0.8 ml 2% bis-acrylamide, 1.9 ml 1.5 M Tris pH 6.6, 75 µl 20% SDS, 10.6 ml distilled water , 75 µl 20% ammonium persulphate, 30 µl TEMED.

TBSTween (0.1%)

1.0 ml of Tween 20 (BDH) dissolved in 1 L of TBS.

Transfer Buffer (10x)

30.3 g Tris and 144.14 g glycine dissolved in 1L of distilled water .

Transfer Buffer (1x)

200 ml methanol, 100 ml 10x transfer buffer (7.1.12) and 1g SDS made upto a 1 L with distilled water .

Tris-buffered saline (TBS)

10 mM Tris pH 7.4 (5 ml 1M Tris pH 7.4), 100 mM NaCl (10 ml 5M NaCl).

MOLECULAR STUDIES

Agarose gel (1%)

1.0 g of agarose in 100 ml of 1x TBE was boiled in a microwave for 30 sec. Once the agarose dissolved it was cooled to 45°C and 3 µl of 10 mg/ml ethidium bromide was added. The gel was then poured into the casting tray, comb placed in position and allowed to set for at least 30 min. The comb was removed from the gel and the gel placed in a tank with sufficient 1x TBE running buffer added to cover it to a depth of 5 mm.

Ampicillin

Dissolved 2.5 g ampicillin into 50 ml of sterile distilled water, followed by filter sterilisation using 0.45 μM filter (ICN). The antibiotic was stored at -20°C.

DEPC water

Added DEPC at a final concentration of 0.01% to ddH_2O and left overnight at room temperature. Following the overnight incubation the DEPC water was autoclaved.

DNA Loading buffer (6x)

50 ml of 50% glycerol, 20 ml of 1 mM EDTA pH8.0, 0.25% bromophenol blue, 0.25% xylene cyanol made up to 100 ml with ddH_2O.

DNA primers

The ß-actin primers were purchased from GIBCO-BRL, the CCK-R primers were synthesised in the department of molecular medicine (King's College School of Medicine and Dentistry) and the K-*ras* primers were purchased from Oswell Company (Southampton).

ß-actin forward primer (5' CCTTCCTGGGCATGGAGTCCTG 3')

ß-actin reverse primer (5' GAGCAATGATCTTGATCTTC 3')

CCK-A forward primer (5' CCTACGACACCGCCTCCGC 3')

CCK-A reverse primer (5' TCCGTTCTTTCTTCTCTGCCTCCT 3')

CCK-B forward primer (5' ACCCCAACGACAGGAAAAGGT 3')

CCK-B reverse primer (5' TTTGGGAAGGAAGGAGAGGGC 3')

K-*ras* exon 1 forward primer (5' -GAGAATTCATGACTGAATATAAACTTGT -3')

K-*ras* exon 1 reverse primer (5' -TCGAATTCCTCTATTGTTGGATCATATTCG -3')

K-*ras* exon 2 forward primer (5' -GCAAGTAGTAATTGATGGAG -3')

K-*ras* exon 2 reverse primer (5' -AGAAAGCCCTCCCCAGTCCT -3')

Enzyme digestion buffers

These buffers were purchased with the respective enzymes from Promega:

Buffer B (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA, 50% glycerol) and Buffer E (10 mM Tris-HCl, pH 7.4, 300 mM KCl,

0.1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA, 50% glycerol)

Ethanol (70%)

70 ml of ethanol was added to 30 ml of sterile water and stored at -20°C.

Glycerol-polyacrylamide gel

5%: 5 ml 10x TBE, 15 ml 40% acrylamide solution (BDH), 5 ml glycerol (BDH), 12.5 ml 2% Bis-acrylamide (BDH), 62.5 ml sterile water, 60 µl TEMED (Sigma) and 60 µl 25% Ammonium persulphate (BDH). 0.5x TBE as running buffer at 3 Watts overnight.

10%: 10 ml 10x TBE, 15 ml 40% polyacrylamide solution, 10 ml glycerol, 12.5 ml 2% Bis, 52.5 ml sterile water, 60 µl TEMED and 60 µl of 25% Ammonium persulphate. 1x TBE as running buffer at 5 Watts overnight.

Hepes-NaOH buffer

130 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES, 1 mM EDTA and 0.125 g/l bacitracin (Sigma).

LB Agar

To 1.0 L of LB broth added 20 g of agar (Difco) and autoclaved. The broth was stored at 4°C.

LB Agar with X-gal and ampicillin

To 1 L LB agar at 55°C added 400 µl 100 mg/ml X-gal (Promega) and 100 µl 50 mg/ml ampicillin.

LB Broth

10 g sodium chloride, 10 g tryptone, 5 g yeast extract and distilled water. Adjust pH to 7 with 5 M sodium hydroxide and autoclaved.

Loading buffer (SSCP)

0.3% bromophenol blue, 0.3% xylene cyanol, 10 mM EDTA and 95% formamide.

Lysis buffer

1%SDS in 0.2 M sodium hydroxide

MOPS (10x)

0.4 M MOPS, 0.1 M sodium acetate, 10 mM EDTA, pH solution to 7.2 with 5 M sodium hydroxide. The solution was autoclaved and stored at 4°C in darkness.

NZY broth

5 g of sodium chloride , 2 g of magnesium sulphate ($7H_2O$), 5 g of yeast extract, 10 g of NZ Amine (Casamino acids), dissolved in 1.0 L of ddH_2O having adjusted to pH 7.5 with 5 M sodium hydroxide. The broth was autoclaved and stored at 4°C.

NZY Agar

5 g of Sodium chloride , 2 g of magnesium sulphate ($7H_2O$), 5 g of yeast extract, 10 g of NZ Amine (Casamino acids), 15 g of agar dissolved in 1.0 L of ddH_2O and autoclaved, stored at 4°C.

One phor all buffer (10x)

100 mM Tris-acetate (pH7.5), 100 mM magnesium acetate and 500 mM potassium acetate.

Polyacrylamide gel (6%)

40 ml of sequagel 6 and 10 ml of sequagel buffer (National Diagnostics), 400 µl 10% ammonium persulphate were mixed and rapidly poured into the gel plates.

3M potassium acetate (pH 4.8)

To prepare 100 ml, mixed 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of distilled water.

RNA Formaldehyde gel

A 1% formaldehyde agarose gel (100 ml) was prepared by boiling 1 g of agarose in 85 ml of sterile water. Once the agarose dissolved it was cooled to 45°C and 10 ml of 10x

MOPS buffer, 5 ml of 40% formaldehyde and 3 µl of ethidium bromide (10 mg/ml) added in a fume cupboard. The solution was mixed thoroughly, poured into a casting tray and allowed to set (30 min). Once set, the comb was removed and the gel placed in the tank with sufficient 1x MOPS running buffer to cover to a depth of 2 mm.

Stripping buffer (10x)

50 mM Tris-HCl pH 8, 2 mM EDTA, 0.5% sodium pyrophosphate, 0.02% polyvinyl-pyrrolidone, 0.02% bovine serum albumin and 0.02% ficoll.

Taq buffer (10x)

This buffer was purchased with Taq polymerase (Promega) 500 mM KCl, 100 mM Tris-HCL pH 9.0, 1% Triton X-100 and 15 mM $MgCl_2$.

TE buffer

1 mM EDTA, 10 mM Tris pH 7.5

Trichloroacetic acid (10%)

5 g of trichloroacetic acid powder (BDH) was dissolved in 50 ml of distilled water. The 10% solution was stored at 4°C in the dark.

Tris-borate EDTA (TBE,10x)

121 g Tris base, 55 g boric acid and 7.45 g EDTA adjusted to 1.0L with d ddH_2O and autoclaved.

TNE (10x)

100 mM sodium chloride, 10 mM Tris pH 8.0 and 1 mM EDTA

Kits

Superscript reverse transcriptase

This kit was purchased from Gibco BRL and stored at -20°C. The kit comprised of the following:

First strand buffer (5x: 250 mM Tris/HCl, pH 8.3, 375 mM potassium chloride, 15

mM magnesium chloride), 0.1 M DTT, mixed dNTP stock and superscript reverse transcriptase.

Qiaex II

This kit was purchased from Qiagen and consisted of the following:

Buffer QX1 (3M sodium iodide, 4M $NaClO_4$, 10 mM Tris/HCl, pH 7.0, 10 mM sodium thiosulphate), Buffer QX2 (8M $NaClO_4$, 10 mM Tris/HCl, pH 7.0), Buffer QX3 (70% ethanol, 100 mM sodium chloride, 10 mM Tris/HCl, pH 7.0) and Qiaex beads.

TA cloning kit

This kit was purchased from In vitrogen and consisted of the following:

Ligation buffer (10x: 0.5 M Tris-Cl pH7.5, 50 mM $MgCl_2$, 50 mM DTT and 0.5 mg/ml BSA), pCR™ vector, 1 u/µl T4 DNA ligase, Competent *E.Coli* cells, 0.5 M β-mercaptoethanol, SOC medium (2% bacto-tryptone, 0.5% bacto-yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulphate and 20 mM glucose).

T7Sequencing kit

This kit was purchased from Pharmacia Biotech and consisted of the following:

Labelling mix A (1.375 µM each dCTP, dGTP and dTTP in 333.5 mM NaCl),

Annealing buffer (1M Tris-HCl (pH7.6), 100 mM MgCl2 and 160 mM dithiothreitol),

Short mixes

'A' (840 µM each dCTP, dGTP, and dTTP; 93.5 µM dATP; 14 µM ddATP; 40mM Tris-HCl (pH 7.6) and 50 mM NaCl)

'C' (840 µM each dATP, dGTP, and dTTP; 93.5 µM dCTP; 17 µM ddCTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl)

'G' (840 µM each dATP, dCTP, and dTTP; 93.5 µM dGTP; 14 µM ddGTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl)

'T' (840 µM each dATP, dCTP, and dGTP; 93.5 µM dTTP; 14 µM ddTTP; 40 mM

Tris-HCl (pH 7.6) and 50 mM NaCl)

Stop buffer (0.3% bromophenol blue and xylene cyanol, 10 mM EDTA pH 7.5, and 97.5% deionised formamide), Universal primer (5'-d[GTAAAACGACGGCCAGT]-3' in aqueous solution, 0.86 A_{260} units/ml (5 pmol/µl)), Enzyme dilution buffer (20 mM Tris-HCl (pH 7.5), 5 mM DTT, 100 µg BSA/ml and 5% glycerol), T7 DNA polymerase (8 u/µl in buffered glycerol solution).

Maxi-script and RPA II kit

These kits were purchased from AMS-biotechnology and consisted of the following: Nuclease free water, Transcription buffer (10x), 2.5 mM of each NTP, rRNasin ribonuclease inhibitor (20 u/µl), SP6 polymerase and T7 polymerase (20 u/µl), RNase-free DNase I (2 u/µl), gel loading buffer (95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.5 mM EDTA, and 0.025% SDS), Probe elution buffer (0.5 M ammonium acetate, 1 mM EDTA and 0.2% SDS), RNase solution (250 units/ml RNase A (approximately 0.5 mg/ml RNase A) and 10,000 units/ml RNase T1), Hybridisation buffer (80% deionised formamide, 100 mM sodium citrate pH 6.4, 300 mM sodium acetate pH 6.4, 1 mM EDTA), Solution Dx (patent pending), and yeast RNA (5 µg/ul).

IN VIVO STUDIES

TNE (10x)

100 mM sodium chloride, 10 mM Tris (pH 8) and 1 mM EDTA.

Sample buffer (protein extraction)

1.1 ml of sample buffer was prepared by adding 200 µl 10% SDS, 240 µl 40% glycerol, 80 µl 1 M Tris-Cl (pH 6.8), 10 µl 0.2% bromophenol blue 100µl of 1 M DTT and 460 µl of sterile water.

PROTEIN KINASE STUDIES

Denaturation buffer

1.5 M NaCl and 0.5 M NaOH

Denhardt solution (50x)

5 g ficoll 400 [2% (w/v)], 5 g polyvinyl-pyrrolidone and 5 g BSA made upto 500 ml with ddH_2O. Filter sterilised and stored at -20°C.

Hybridisation buffer

4 ml formamide, 0.8 ml 50x Denhardts, 200 µl 20% SDS, 2.4 ml 20% SSC, 80 µl boiled 10 mg/ml salmon sperm DNA and 333 µl sterile ddH2O.

Neutralisation buffer

1.5 M NaCl and 0.5 M Tris pH8.0

NZY Agar -kanamycin

1 L of NZY agar autoclaved, cooled to 55°C with 50 mg of filter-sterilised kanamycin added. The fresh agar is poured into petri-dishes prior to use.

Oligo-labelling buffer (OLB)

OLB is made up by mixing the solutions A:B:C in a ratio of 10:25:15.

Solution A: 1.25 M Tris-HCl (pH 8), 0.125 M MgCl (pH 8), 10 mM ß-mercaptoethanol, 0.5 mM dATP, 0.5 mM dGTP and 0.5 mM dTTP.

Solution B: 2 M Hepes (pH 6.6)

Solution C: Oligo dTTTTs. Dissolve 50 optical density units of powder in 0.55 ml T.E.

Phage Buffer

5.8 g sodium chloride, 2.0 g magnesium sulphate, 50 ml 1M Tris-HCl (pH 7.5), 0.1 g gelatin made upto 1 L with ddH_2O.

PTK Primers

These primers were purchased from King's College School of Medicine and Dentistry:

sense 17MER: 5'-CGGATCCAC(A/C)G(A/C/G/T)GA(C/T)(C/T)T-3'

antisense 23MER: 5'-GGAATTCCA(A/T)AGGACCA(G/C)AC(A/G)TC-3'

Salmon sperm DNA

Salmon sperm was dissolved in ddH_2O at a concentration of 10 mg/ml and autoclaved for 10 min at 121°C. Sodium chloride was added to a final concentration of 0.1 M and the DNA extracted with an equal volume of phenol/chloroform (1:1), followed by chloroform extraction prior to alcohol precipitation (2.5 volumes). DNA was then resuspended in T.E to a final concentration of 10 mg/ml and stored at -20°C.

Sephadex G50

100 g of sephadex beads were added to 100 ml of ddH_2O, after vigorous mixing the solution was topped up with more ddH_2O to a final volume of 200 ml and autoclaved. The sephadex solution was stored at room temperature.

SSC (20x)

175.3 g of sodium chloride and 88.2 g of sodium citrate dissolved in 1 L ddH2O and adjusted to pH 7.0.with sodium hydroxide.

Stripping buffer (10x)

50 mM Tris-HCl pH 8, 2 mM EDTA, 0.5% sodium pyrophosphate, 0.02% polyvinyl-pyrrolidone, 0.02% bovine serum albumin and 0.02% ficoll.

Top agar

0.7% (w/v) agarose was added to 1 L of NZY agar and autoclaved.

Kits

Zap express^{TM} predigested vector kit

This kit was purchased from Stratagene and consisted of the following:

vector phage arms (1 µg/µl), Ligase buffer (10x: 300 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 100 mM DTT and 10 mM ATP), and T4 DNA ligase (8 u/µl).

APPENDIX II

Tumour weight (g)					
Control	**DMSO**	**NH4OH**	**Saline**		
0.04	0.875	0.05	0.151		
0.044	0.059	0	0.196		
1.426	0	0	0.061		
0	0.397	0	0.887		
0.815	0.493	0.07	0.32		
0	0.137	0.199	0.215		
0.42	0				
1.866	0				
0.52	0.666				
0.116	0.13				
0.251	0.328				
0.280	0.676				
mean	**0.48**	**0.313**	**0.054**	**0.305**	
	L-365 260	**L-364 718**	**L-740 093**	**RPR-X**	**JB93182**
	0.27	0.311	0.851	0.098	0.117
	2.86	0.632	0.977	0.258	0
	0.771	0.553	0.311	1.503	0
	0	0.137	0.607	0.516	0.610
	0.365	1.724	2.81	0	0
	0.270	0	0.139	0.218	0
	0.212	0.44	0.138	0.277	
	0	0.114	0	0.094	
	0.418	0.016	0.197		
mean	**0.574**	**0.436**	**0.670**	**0.371**	**0.125**
			sCCK-8 (µg/kg)		
		in DMSO			in NH_4OH
	15	**30**	**100**	**15**	**30**	**100**
	0.294	0.044	0.464	0.997	1.186	0
	1.497	1.35	2.098	0.135	0.031	0
	0	1.206	0.22	0.512	0	1.029
	0.144	0.576	0.236	0.193	0	1.18
	0	0	0.231	0.021	0	0.878
	0	0	0.224	0.869		0
mean	**0.323**	**0.529**	**0.579**	**0.455**	**0.241**	**0.515**
			nsG-17 (µg/kg)			
		in DMSO			in NH_4OH	
	15	**30**	**100**	**15**	**30**	**100**
	0	0.144	0.901	0.961	0.248	0.385
	0.338	0.293	0.135	0.523	0.163	0.277
	0.93	0.178	1.671	1.042	0.165	0.375
	0.018	0.103	2.263	0	0.565	0.255
	1.456	0.506	0.213	0.117	0.203	0.292
		0.255		0.09	0.511	1.6
mean	**0.548**	**0.247**	**1.037**	**0.456**	**0.309**	**0.537**

Table 1 Showing individual weights of the treated and untreated xenografted tumours.

	Tumour weight (g)				
	Control	**DMSO**	**NH4OH**	**Saline**	
average	0.48	0.313	0.054	0.305	
median	0.27	0.23	0.025	0.21	
SD	0.603	0.307	0.076	0.297	
sem	0.174	0.089	0.032	0.121	
CV	125.3%	97.9%	145.8%	97.5%	
	L-365 260	**L-364 718**	**L-740 093**	**RPR-X**	**JB93182**
average	0.574	0.436	0.67	0.371	0.125
median	0.27	0.311	0.311	0.238	0
SD	0.888	0.534	0.872	0.483	0.244
sem	0.296	0.178	0.291	0.171	0.1
CV	155%	123%	130%	130%	201%

	sCCK-8 (μg/kg)					
	in DMSO			in NH4OH		
	15	**30**	**100**	**15**	**30**	**100**
average	0.323	0.529	0.579	0.455	0.241	0.515
median	0.072	0.31	0.23	0.35	0	0.44
SD	0.587	0.621	0.75	0.407	0.527	0.572
sem	0.24	0.254	0.306	0.166	0.236	0.233
CV	182%	117%	129.6%	89.5%	217%	111%

	nsG-17 (μg/kg)					
	in DMSO			in NH4OH		
	15	**30**	**100**	**15**	**30**	**100**
average	0.548	0.247	1.037	0.456	0.309	0.537
median	0.338	0.22	0.901	0.32	0.23	0.33
SD	0.632	0.145	0.924	0.46	0.181	0.547
sem	0.282	0.059	0.413	0.188	0.074	0.222
CV	115%	58.9%	89.2%	101%	58.5%	101%

Table 2 Showing the calculated averages, medians, standard deviations (SD), standard error of the mean (sem) and coefficient of variation (CV) of the treated and untreated xenografted tumour weights.

	DNA concentration (mg/kg of tumour)			
	Control	**DMSO**	**NH4OH**	**Saline**
	1.0	0.91	0.95	0.99
	0.9	1.02	0.89	1.07
	0.98	0.88	1.01	1.18
	0.99	1.01		0.97
	0.95	0.95		0.99
	0.96	0.88		0.93
	0.94	0.923		
	0.91	0.973		
	0.99	1.01		
	1.07			
mean	**0.97**	**0.95**	**0.95**	**1.02**

	L-365 260	**L-364 718**	**L-740 093**	**RPR-X**	**JB93182**
	0.96	0.97	0.93	1.02	0.92
	0.94	0.94	1.02	0.85	1.10
	1.04	0.94	0.93	0.99	
	0.98	0.95	0.94	0.95	
	1.00	1.04	0.98	1.0	
	0.94	0.91	0.93	0.94	
	0.91	0.96	0.94	0.96	
		1.2	1.00		
mean	**0.97**	**0.99**	**0.96**	**0.96**	**1.01**

	sCCK-8 (μg/kg)			**nsG-17 (μg/kg)**		
	15	**30**	**100**	**15**	**30**	**100**
	0.99	1.12	0.86	1.23	1.04	0.99
	0.97	0.89	1.00	1.01	0.86	1.00
	1.0	0.96	0.91	0.98	1.02	1.00
	0.98	0.99	0.85	0.99	1.06	0.99
	0.95	0.95	0.91	0.89	1.01	0.98
	0.85	0.89	0.98	0.85	1.02	0.96
	0.87		1.23	0.86	0.82	0.85
	0.88		0.89	0.88	0.90	0.93
	0.90		0.85	0.96	0.85	0.89
					0.86	0.92
					0.82	1.01
					0.86	1.00
mean	**0.93**	**0.97**	**0.94**	**0.96**	**0.93**	**0.96**

Table 3 Showing individual DNA concentration of the treated and untreated xenografted tumours.

	Protein concentration (mg/kg of tumour)					
	Control	**DMSO**	**NH4OH**	**Saline**		
	89.25	88.00	90.10	87.40		
	89.70	84.80	85.60	87.80		
	87.70	78.34	86.20	88.50		
	86.30	87.22		84.60		
	86.90	87.60		84.36		
	89.20	88.20		93.02		
	86.70	90.20				
	89.60	88.40				
	89.20	89.20				
	89.20					
mean	**88.38**	**86.88**	**87.30**	**87.61**		
	L-365 260	**L-364 718**	**L-740 093**	**RPR-X**	**JB93182**	
	89.00	86.80	91.70	87.80	89.80	
	87.40	87.00	80.14	77.50	86.90	
	86.90	81.30	86.80	87.80		
	87.60	89.10	84.10	89.00		
	88.90	88.70	89.00	91.50		
	89.10	88.60	89.20	89.50		
	88.50	89.50	89.00	85.10		
		87.50	89.30			
mean	**88.20**	**87.31**	**87.41**	**86.89**	**88.35**	
	sCCK-8 (µg/kg)			**nsG-17 (µg/kg)**		
	15	**30**	**100**	**15**	**30**	**100**
	85.00	89.01	87.50	90.30	87.50	85.50
	84.80	86.40	85.80	87.20	82.00	88.90
	90.00	86.70	86.40	87.50	85.40	87.43
	83.33	87.30	79.20	87.30	87.50	88.40
	89.62	88.40	86.50	86.50	89.25	84.00
	90.68	90.20	89.80	88.00	86.30	95.00
	80.52		92.62	88.00	90.20	82.00
	82.00		90.30	86.30	85.00	82.50
	89.00		85.36	89.90	89.50	88.60
					86.30	82.30
					80.00	85.60
					89.20	84.20
mean	**86.11**	**80.00**	**87.05**	**87.89**	**86.51**	**86.20**

Table 4 Showing individual protein concentration of the treated and untreated xenografted tumours.

	Spleen weight (g)				
	Control no tumour	**Control** tumour	**DMSO**	**NH4OH**	**Saline**
	0.117	0.117	0.136	0.125	0.120
	0.152	0.111	0.140	0.110	0.107
	0.123	0.185	0.123	0.122	0.098
	0.113	0.130	0.216	0.113	0.114
	0.146	0.110	0.084	0.112	0.125
	0.115	0.100	0.121	0.115	0.082
		0.154	0.102		
		0.105	0.100		
		0.160	0.101		
		0.160	0.102		
		0.170	0.108		
		0.170	0.106		
mean	**0.128**	**0.139**	**0.120**	**0.116**	**0.108**
	L-365 260	**L-364 718**	**L-740 093**	**RPR-X**	**JB93182**
	0.130	0.130	0.142	0.106	0.080
	0.265	0.088	0.119	0.102	0.120
	0.214	0.121	0.163	0.103	0.085
	0.091	0.091	0.128	0.127	0.161
	0.098	0.090	0.208	0.129	0.180
	0.173	0.098	0.102	0.257	0.130
	0.144	0.075	0.093	0.186	
	0.120	0.166	0.112	0.103	
	0.138	0.09	0.083		
mean	**0.153**	**0.105**	**0.128**	**0.139**	**0.126**

	sCCK-8 (µg/kg)					
	in DMSO			in NH_4OH		
	15	**30**	**100**	**15**	**30**	**100**
	0.140	0.134	0.139	0.152	0.123	0.107
	0.140	0.109	0.217	0.081	0.165	0.122
	0.120	0.157	0.119	0.165	0.100	0.144
	0.136	0.120	0.113	0.191	0.105	0.120
	0.097	0.110	0.100	0.067	0.132	0.170
	0.130	0.103	0.100	0.064		0.100
mean	**0.127**	**0.122**	**0.131**	**0.120**	**0.125**	**0.127**

	nsG-17 (µg/kg)					
	in DMSO			in NH_4OH		
	15	**30**	**100**	**15**	**30**	**100**
	0.103	0.140	0.197	0.100	0.100	0.103
	0.106	0.140	0.177	0.103	0.101	0.099
	0.193	0.164	0.139	0.100	0.134	0.130
	0.200	0.121	0.192	0.150	0.114	0.139
	0.170	0.155	0.084	0.133	0.133	0.102
		0.119		0.132	0.102	0.143
mean	**0.154**	**0.140**	**0.158**	**0.120**	**0.114**	**0.119**

Table 5 Showing individual spleen weights of the treated and untreated mice.

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